NOV 3 1948

Official Organ of the Society for the Study of Development and Growth

GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

PERIODICAL ROOM
GENERAL LIBRARY
UNIV. OF MICH.

VOLUME XII, NUMBER 3

PAPERS 337 - 340

SEPTEMBER, 1948 **$5.00 A YEAR**

Entered as second class matter on October 20, 1941, at the Post Office, Worcester, Mass., Under the Act of March 3, 1879.

GROWTH

A journal for the coördination of studies of increase and development as general properties of nature. Published by contributors and subscribers on a coöperative, non-profit, non-salary basis. The field of investigation is primary and work will be published in which the approach is through mathematics, physics, chemistry, genetics, embryology, philosophy, and anthropology; just as will studies of development in its several expressions, and of mass increase and the part played therein by nutrition, vitamines, and hormones.

The journal will appear regularly at quarterly intervals. Subscription is $5.00 per volume (outside U. S. and Canada, $5.50). Single numbers $2.00. There will be one volume of at least 400 pages each year.

Subscriptions, manuscripts submitted for publication, and all correspondence should be sent to *The Editors of Growth, Lankenau Hospital Research Institute, Girard and Corinthian Avenues, Philadelphia 30, Pa.*

THE EDITORS OF GROWTH

John Berrill, McGill University; S. Brody, University of Missouri; H. S. Burr, Yale University; S. A. Courtis, University of Michigan; C. H. Danforth, Stanford University; L. K. Frank, Macy Foundation; P. W. Gregory, University of California; F. S. Hammett, Lankenau Hospital; Leigh Hoadley, Harvard University; Clyde Kluckhohn, Harvard University; Warren H. Lewis, The Wistar Institute; Leo Loeb, Washington University; Carroll E. Palmer, U. S. Public Health Service; Otto Rahn, Cornell University; H. S. Reed, University of California; S. P. Reimann, Lankenau Hospital; R. E. Scammon, University of Minnesota; E. W. Sinnott, Yale University; K. V. Thimann, Harvard University; Paul Weiss, University of Chicago; Philip R. White, Institute for Cancer Research, Philadelphia; D. M. Whitaker, Stanford University; B. H. Willier, Johns Hopkins University.

Corresponding Editors

B. Boysen Jensen, University of Copenhagen; Ivar Broman, Tornblad Institutet; Luigi Castaldi, Università di Cagliari; A. Dalcq, Université de Bruxelles; E. Fauré-Fremiet, College de France; Julian S. Huxley, Zoölogical Society; J. Krizenecky, Vysoka Skola Zemedelska; Joseph Needham, Caius College; Yo. K. Okada, Kyoto Imperial University; G. Teissier, Faculté des Sciences.

Growth, 1948, **12**, 165-180.

ON SEGMENT FORMATION IN NORMAL AND REGENERATIVE GROWTH OF EARTHWORMS

G. E. GATES

(Received for publication March 15, 1948)

Earthworms are said to form new segments continuously. Presumably that means throughout all of the postembryonic life. Earthworms are also said to hatch from the cocoons with a full complement of segments. Both statements are usually made without any qualification of the word "earthworms." Do or do not earthworms lay down new segments after hatching?

The present contribution attempts to answer that question with regard to representative species of several families. In addition, as tail regeneration is thought to be practically identical with normal growth, in microscopic as well as macroscopic detail (Hyman, 1940, p. 521), some observations on segment formation in tail regeneration are also presented.

TERMINOLOGY AND METHODS

Juvenile is used for all stages after hatching except those mentioned below. Characterizations such as recently hatched are estimates, as life histories of earthworms are only partly known or are quite unknown. Size designations appear to be the most satisfactory method, for the time being, of characterizing these stages.

A later stage in which the worms have certain stigmata of maturity such as tubercula pubertatis, genital markings, porophores, etc., but no clitellum is designated aclitellate.

Individuals with externally recognizable epidermal thickening on clitellar segments are termed clitellate. The clitellum, at least in the Megascolecidae, when recognizable, usually and normally extends to definite limits characteristic for species or genus.

Aclitellate specimens with clitellar segments marked by a more or less dark brownish colouration are referred to as postsexual to distinguish them from presexual worms without the discolouration.

For convenience, growth is considered under three designations:

embryonic, postembryonic (demarcated by hatching), and regenerative.

Regeneration may take place in nature as well as in the laboratory. The two are distinguished, for convenience only, as natural and experimental. Amputation is used in a very general sense for loss of a portion of the body regardless of cause and may further also be characterized as natural and experimental.

Measurements were made on worms so preserved as to be uniformly contracted. Uniform relaxation seems to be more difficult to obtain.

An ideal program requires breeding of correctly identified worms under conditions that permit counting segments on particular individuals at various stages of the life history. Two species were successfully bred in the laboratory, but circumstances prevented growth to maturity. Pending more favorable conditions series of various size stages were collected as opportunity offered and later studied.

Data as to external characteristics of posterior ends were obtained under highest powers of the dissecting binocular, in the most brilliant illumination available. Failure to use such magnification and illumination may have been responsible for previous inability to recognize certain characteristics that now seem fairly obvious.

Most of the results presented were secured in India, in wartime conditions, of constantly increasing difficulty which finally compelled abandonment not only of the project but also of a very considerable amount of unstudied material.

Family Megascolecidae

Genus *Perionyx* E. Perrier

Perionyx sansibaricus Michaelsen 1891

This species was found, in a year round survey involving identification of more than fifteen thousand specimens (Gates, 1945), to be the only one of its genus in the fauna of Allahabad. Because of a characteristic red pigment it is distinguishable at a glance from all other Allahabad species. Furthermore, other external characteristics, clearly recognizable even in the smallest juveniles, enable ready distinction not only from all other Allahabad worms but also from another species of the nearest locality in which the same genus is again represented.

Clitellate specimens were collected, individually identified and then kept in conditions permitting deposition of cocoons while guaranteeing absence of infection by other species. Cocoons were isolated in con-

tainers permitting examination without opening. In the laboratory worms hatched out in 9-18 days. After hatching each individual was identified specifically before further study.

In each just-hatched juvenile the posterior-most portion of the body, containing the terminal anus, was smooth-surfaced, without any trace of furrowing. Length of this zone varied considerably but was always very much longer than that of a single segment. Next in front, was a zone in which more or less completely circumferential furrows were present, the anterior more clearly marked and often with anlage of dorsal pores, the posterior fainter, without pore anlage and often incomplete. Those furrows apparently marked off rudimentary segments of nearly uniform size, in which setae were unrecognizable externally. In an antepenultimate zone, segments were very slightly larger and setae were present, in complete circles anteriorly, incomplete posteriorly. In a fourth zone, segments were relatively small for some distance forwards.

In the penultimate zone there may be more furrows than septa. Some of these furrows, accordingly, may not be rudimentary intersegmental furrows. Determination of those furrows which do correspond to internal septa appears impossible without sections. In these circumstances it seemed advisable to count as segments only those axial portions that could be recognized as such by external examination. Presence of externally recognizable setae provided the desired certainty.

Numbers of setigerous segments in the just hatched worms, 6-10 $\times$ 0.5-0.75 mm., of 35 specimens was as follows: 53 (1 specimen), 61 (1), 64 (1), 66 (1), 67 (1), 68 (1), 69 (2), 70 (3), 71 (2), 72 (2), 73 (5), 74 (4), 75 (4), 76 (2), 77 (3), 78 (1), 82 (1). Including non-setigerous but probably definitely demarcated segments, and even axial portions of the body doubtfully delimited as segments, total numbers in each specimen were always less than one hundred.

Somewhat older juveniles were obtained from the breeding grounds. In a series with a size range (length $\times$ thickness) of 14-25 $\times$ 1.0-1.75 mm., number of setigerous segments, after screening out all amputees, varied from 85-133. In another series, presumably somewhat older, with a size range of 26-35 $\times$ 2.0-2.5 mm. number of setigerous segments varied, again after screening out all amputees, from 118-153. Every individual in each of those two series had, in the posterior por-

tion of the body, the same four regions as the just-hatched juveniles, and again the terminal region was very much longer than a single segment.

In larger juveniles, of various sizes, there was a definite tendency for the unpigmented and unfurrowed posterior zone to be shorter, absolutely as well as relatively. Exceptions, however, were not rare. Some clitellate worms, actually functioning sexually, had the same four zones as just-hatched juveniles. Usually percentage of such individuals was small, but in one fairly long series obtained in April (B), more than 80% of the specimens were so characterized.

In all other clitellate specimens, after exclusion of amputees (unregenerate as well as regenerating and regenerated), although some slight and gradual decrease in segment size was still recognizable posteriorly, metameric differentiation appeared at first to be complete. More careful examination of that small anal region which at first appeared to be a single segment enabled distribution of the specimens into eleven classes with the characteristics mentioned below.

1. Anal region without nephropores and setae. On the dorsum slightly behind the intersegmental furrow forming the anterior boundary, a transverse line of pigment of about the same appearance as the pigment in the segment in front.

2. As in 1 but pigment band noticeably wider, i.e., longer anteroposteriorly, more ribbon or band-like.

3. As in 2 but with a pair of nephropores present just behind the anterior intersegmental furrow.

4. As in 3 but with setae present, either a few ventrally only, or a larger number in an arc extending dorsally, or (very rarely) a complete or nearly complete circle

5. A very slight, transverse furrow behind pigmented portion, either on the dorsum or the ventrum. When the furrow was dorsal there might be visible at the mid-dorsal line a minute, greyish rudiment of a dorsal pore.

6. As in 2 but pigmented band succeeded anteroposteriorly by a narrow unpigmented region and a fine transverse line of pigment.

7. As in 6 but with posterior pigment in a band not quite as wide as the anterior band.

8. As in 7 but with one pair of nephropores in region of anterior pigment band.

9. As in 7 but with a few setae ventrally in region of anterior pigmented band.

10. Regions of both bands of pigment provided with a few ventral setae, or with nephropores.

11. A very slight transverse furrow present ventrally between the two setigerous regions of class 10. By extension of that furrow dorsally on each side so as to become completely circumferential a small segment would be formed.

The first class contained by far the largest number of specimens and classes 6-11 had only a very few specimens each.

Counting as one segment the terminal anal region in each of the eleven classes, number of segments of 449 April specimens from Allahabad (A) was found to be 139-197 (Table I). In specimens (200 +) secured in other months, number of segments varied from 101-200. In large collections from Jubbulpore a maximum was also reached at 200.

Postsexual aclitellate specimens, after clitellar regression, as well as late presexual aclitellates, also had in the small terminal anal region the same characteristics as clitellate worms.

TABLE I

NUMBER OF SEGMENTS IN CLITELLATE SPECIMENS OF *P. sansibaricus*

S	♯	S	♯	S	♯
139	4	159	14	179	2
140	3	160	15	180	5
141	2	161	16	181	2
142	10	162	13	182	5
143	9	163	14	183	1
144	5	164	8	184	5
145	12	165	16	185	1
146	14	166	10	186	—
147	12	167	13	187	—
148	6	168	10	188	—
149	13	169	10	189	4
150	10	170	8	190	1
151	17	171	12	191	1
152	15	172	7	192	—
153	13	173	12	193	—
154	17	174	6	194	2
155	16	175	3	195	1
156	9	176	2	196	—
157	15	177	5	197	1
158	13	178	9	198	—

S Number of Segments ♯ Number of Specimens

Number of segments previously recorded: 108 (Michaelsen, 1900, p. 210), 84-108 (Stephenson, 1923, p. 356).

Discussion

It seems fairy clear, even without microtome sections, that the relatively large, terminal anal zone of just-hatched juveniles must be a region primarily of cell division and cell growth. A penultimate zone, as indicated by the more or less rudimentary intersegmental furrows, is one where demarcation of segments begins, while the antepenultimate zone is one where metameric differentiation is completed. The fourth zone is equally clearly a region of segment growth.

Every juvenile as it emerged from the cocoon accordingly had what may be called a growth region in which four zones, each with its own characteristics, were recognizable. No exceptions were found to this rule.

Somewhat older juveniles hatched in natural conditions in the breeding grounds, with exception of amputees (with or without regenerates), always had similar growth zones. That presumably indicates presence of similar zones in the just-hatched juveniles was not due to premature hatching in the laboratory (Sun and Pratt, 1931, p. 42).

The size of the zones, numbers of parts therein, or in case of terminal zone the number potentiality as indicated by size, give an appearance of production of a number of segments almost simultaneously. Presumably in such a growth region formation of new segments is a relatively rapid process.

Presence, in older juveniles 2½-3½ times the length of those just hatched, of the very same growth zones, seems to indicate that a postembryonic period of rapid segment formation continues for some time. Comparison of segment numbers in those older juveniles and in clitellate worms apparently indicates that the great majority of segments are produced during that early period of rapid segment formation (*vide* Table II).

TABLE II

Segment Number and Body Size in *Perionyx sansibaricus*

Size in mm.			Number of	
Length	Diameter	Condition	Segments	Comments
6-10	0.5-0.75	Just Hatched	53-82	Only
14-15	1.0-1.75	Juvenile	85-133	setigerous
26-35	2.0-2.5	Juvenile	118-153	segments counted
-160*	-5*	Clitellate	101-200	Anal Region Counted as 1 Segment

*New records.

In certain conditions a worm may become mature and function sexually while still in possession of a juvenile growth region. Eventually, however, the last two zones are reduced to a very small region regarded hitherto usually as a single anal segment. In that small terminal region there are recognizable rudiments of one or two segments only. The small size of this growth region, its appearance of one-at-a-time, or at most two-at-a-time, formation of segments, the slight difference between the stages and the relative rarity of all but the earliest, suggest that rate of segment production is slow.

Especially significant seems to be the fact that, after exclusion of amputees and regardless of stage, i.e., whether clitellate or pre- or postsexual aclitellate, every specimen has in its anal region rudiments of one or two or occasionally even more, segments. Unless growth processes are irreversibly inhibited while segments are still very rudimentary, there is always a possibility of addition of one, two or more segments by completion of metameric differentiation already begun. In such circumstances "continuous" production of new segments, throughout life, appears theoretically possible.

Theoretically perforce had to be used because of lack of knowledge of life histories of earthworms. *P sansibaricus* in favorable conditions does survive at least one breeding period. Distinction, at least by external characteristics of a later from a first sexual period appears to be impossible.

After operation and in laboratory conditions, certain Lumbricid species have lived four to ten years (Korschelt, 1914). *P. sansibaricus* which is of about the same size and lives in a somewhat similar habitat may perhaps be expected to survive as long as *Eisenia foetida* which has a theoretical expectancy of 4 + years.

During such a period segment formation could be prevented by amputation, breeding or diapause. Unpublished data (to be discussed elsewhere) indicate that the longer the worm lives the greater the likelihood of losing more or less of the posterior part of its body, including of course the growth region. Further segment formation would then be contingent on ability to regenerate a growth zone. Some species of earthworms apparently are unable to do that. In other species an occasional individual seems unable to regenerate.

Breeding probably is possible, in proper conditions, throughout the year, but lengths of individual breeding periods and of non-repro-

ductive intervals are unknown. Effect of tropical seasonality on segment production also remains to be studied. At present *sansibaricus* does not appear to have an obligatory diapause, i.e., a resting period imposed by its own physiological organization independent of external factors. However in all of its original range a diapause lasting from one to several months is imposed by lack of moisture in those regions of monsoon rainfall.

In another species of the genus *Perionyx, P. excavatus* E. Perrier, 1872, presence in sexually mature individuals of a juvenile growth region has already been indicated (Gates, 1941, p. 182). Records for that species lost in Rangoon probably contained considerable data of the same sort as presented above for the Indian species.

Tail regeneration, in *P. sansibaricus,* because of extent of accumulated data, is reserved for consideration elsewhere. However, it may be noted here that size of the growth region may vary according to age and level and further at a particular level and age in accordance with unknown conditions. Eventually, as in normal worms, the larger growth region of rapid segment formation is reduced to the very much smaller region of slow segment formation. Here, again, there seems to be a theoretical possibility of continuous growth.

Genus *Lampito* Kinberg
Lampito mauritii Kinberg 1867

This species also was found (Gates, 1945) to be the only one of its genus in Allahabad and vicinity. Not for a number of hundreds of miles is there another species. In such conditions juveniles of *mauritii,* even though very small and quite recently hatched, can be specifically identified without difficulty. Although the species survives laboratory conditions fairly well no cocoon deposition was observed and all material had to be obtained from the breeding grounds.

The smallest juveniles, less than a millimeter in diameter, obtained with considerable difficulty, already had 70-80 setigerous segments. Larger juveniles, presumably several weeks older, of a size range 20-30 $\times$ *ca.* 1 mm, had 100-135 setigerous segments. Each one of those juveniles had posteriorly a growth region with the same four zones as in *P. sansibaricus.*

Occasionally presexual aclitellate, very rarely even sexually functioning individuals, were found to have the same growth zones.

Number of segments in clitellate specimens, after exclusion of all amputees, was 100-189.

Comments.—Size of the youngest juveniles seems to indicate a short embryonic period.

This species, like *P. sansibaricus,* appears not to have an obligatory diapause. The usual habitat is distinctly different from that of *sansibaricus* and dries up earlier. The climatically enforced resting period, which may be in winter or summer according to rainfall or irrigation, usually would be longer than for *sansibaricus.*

Several thousand specimens with natural tail regenerates of various sizes, and presumably of various ages, were collected. Each regenerate had a terminal anus, and a growth region, large or small, as in *P. sansibaricus.*

Family Lumbricidae
Genus *Eisenia* Malm
Eisenia foetida (Savigny) 1826

The youngest juveniles secured were of unknown age and had been without food since hatching. Size and number of segments is shown below. Juvenile growth zones as in *P. sansibaricus* and *L. mauritii* were lacking and the condition of the small anal region was similar to that of the adult specimens characterized below.

TABLE III
SEGMENT NUMBER AND BODY SIZE IN JUVENILES OF EISENIA FOETIDA

	Length and Diameter in mm.		Number of Segments
1.	7.5	0.9	85.
2.	8.	0.9	111
3.	10.	1.5	105
4.	12.	1.5	114
5.	15.	1.5	102
6.	16.	1.75	91

These specimens were strongly and apparently uniformly contracted.

As in case of *P. sansibaricus* a small and terminal anal region at first appeared unpigmented and with no indications of growth. Again more careful examination revealed in every specimen, whether large juvenile, presexual and postsexual aclitellate, clitellate, indications of a small growth zone. All of the specimens could be distributed into five classes.

1. Pigment present on dorsum of anal region in a fine transverse line slightly behind the intersegmental furrow.
2. Pigment in a fairly broad, ribbon-like band.
3. As in 2 but setae present ventrally.
4. Four setae present ventrally in an anterior portion of anal region but no pigment recognizable dorsally.
5. As in 3 but with indication of a rudimentary intersegmental furrow behind region of setae and pigment. When furrow was dorsal a dark greyish spot at mid-dorsal line, in the furrow, represented a rudimentary dorsal pore.

The majority of the specimens were in the first two classes, the last class with only four specimens out of a large number examined.

Number of segments in clitellate specimens was in limits of numerous previous records, with a maximum of 125.

In young, natural, tail regenerates at levels from 42/43 to 52/53 the anus usually was a longitudinally placed aperture, wholly on the dorsal side, not even reaching to the posterior end of the body for the posterior margin was thickened to form a transversely ellipsoidal lobe that was also wholly dorsal. On the ventral side of the anal portion of the regenerate there were a number of closely crowded, arc-shaped intersegmental furrows fading out dorsally.

At levels from 52/53 to 68/69 the anus was occasionally dorsal but a posterior lobe was lacking. In those at 69/70 to 84/85 the anus was partly dorsal and partly posterior, i.e., dorsoterminal. The single case at 84/85 was on a medium-sized juvenile. In all regenerates at levels behind 84/85, and in all old regenerates at other levels, the anus was completely posterior (terminal)

Comments.—The embryonic period of this species is short, about three weeks. In favorable conditions breeding may take place all the year round. Lengths of individual breeding periods and of non-reproductive intervals are unknown. Nothing is known as to diapause.

Liebmann, who bred and raised *E. foetida* in the laboratory reported that in just-hatched juveniles the terminal region "is largely in an undifferentiated embryonic condition" (1943, p. 605). Number of segments apparently was not determined but by six months, according to the same author, in most specimens was somewhat over one hundred (p. 600).

Genus *Lumbricus* L.
Lumbricus terrestris L.

Less than a hundred specimens, mostly clitellate, were available for examination. In each of those worms, in the relatively small and apparently quite unpigmented anal region, no indications of differentiation of new segments were recognized.

In a number of the worms the anal region had an appearance quite similar to that of the anally sculptured cicatrix of *P. sansibaricus*. However, no evidence of regeneration posteriorly was found.

Remarks.—In view of the absence of all external indication of segment formation in the anal region the condition of the nerve cord in the supposedly single anal segment is of especial interest. In that "segment" there are five or six pairs of nerves instead of the three pairs normal for segments throughout the body (Hess, 1925, p. 243). The anterior three pairs correspond in structure to the set of three nerves found in each segment ordinarily. The fourth pair, however, corresponds to the first pair of a segmental set while the last pair (or pairs) is of a quite different sort, lacking ventral branches. Whether these last pairs are still more embryonic or are peculiar to the anal region proper as distinct from its anterior growth portion presumably depends on the method of growth in the cord, i.e., whether strictly terminal or more or less subterminal as in the body as a whole. Length of embryonic development seems to be unknown and a diapause may be entirely lacking. No records of tail regeneration by this species have been found in the literature.

Family Glossoscolecidae
Genus *Glyphidrilus* Horst
Glyphidrilus ceylanensis Gates 1945

Specimens 180-280 mm. long and presumably nearly grown though aclitellate have a dorsal, triangular anus with apex posteriorly. The posterior margin is thickened and has a transversely ellipsoidal shape.

In rather young tail regenerate the anus is dorsoterminal with the lobe at the ventral end of the aperture.

Glyphidrilus papillatus (Rosa) 1890

In young tail regenerates the anus is vertically placed on the posterior face. The ventral margin is thickened and with a rather

spheroidal shape. In older regenerates the anus is wholly dorsal and the thickening of the posterior margin has an ellipsoidal shape.

Comments.—Very little is known of the life history of these or other species of the genus *Glyphidrilus*. Cocoons, just hatched and quite young juveniles have been unobtainable. Evidence available seems to indicate that the dorsal position of the anus is constant throughout postembryonic life. In that position, the anal region has the same morphological relationship to a number of segmental rudiments simultaneously as in certain growth stages of some tail regenerates in *E. foetida (q.v.)*.

In this connection relationship of number of segments relative to length is of interest. In three species, having a maximal length of 140-175 mm., maximal number of segments is 310, 322 or 330.

Length of embryonic development is quite unknown and in absence of recently hatched juveniles cannot even be estimated.

In a permanently aquatic environment an obligatory diapause presumably is lacking but worms living in mud at bottom of temporary waters may have a diapause imposed by periods of tropical drought.

Several other aquatic Glossocolecids, *Alma nilotica* Grube 1855, *Criodrilus lacuum* Hoffmeister 1845, and *Sparganophilus tamesis* Benham 1892, have in the adults, and presumably also in younger stages, a dorsal anus. In those species maximal size and segment number are as follows: *A. nilotica,* 350 × 5 mm., 480 segments; *C. lacuum,* 320 × 5 mm., 450 segments; *S. tamesis,* 138 mm., 214 segments.

Discussion

In all of the species studied, with but one exception, after exclusion of amputees, every specimen, whether juvenile, presexual aclitellate, clitellate or postsexual aclitellate, was found to have a growth region with rudiments of one to several segments more or less readily recognizable externally. Even in the exceptional species the nerve cord of the anal region (Hess) seems to have one or two rudimentary neuromeres. Segmental counts on taxonomically identified individuals of various stages in two of the species show that rudiments do become differentiated into metameres. Segment formation accordingly appears to be involved in postembryonic growth.

Place of origin of new segments was thought by Sun and Pratt (1931, p. 32) to be disputed. The supposed difference now appears

to have been only semantic. In the anterior portion of the anal region there are differentiated those structures and conditions that are characteristic of a typical metamere. That portion is finally marked off by development of an intersegmental furrow externally and of a septum internally. As a result a new segment is intercalated between the anal region and the rest of the body.

A reference to the laying down of segments in a growth zone in front of "the anal segment" (Hyman, 1940, p. 522) may also lead to misunderstanding. In young juveniles and tail regenerates the strictly terminal or anal portion lacks such characteristics of a segment as setae, nephropores, pigment, and more important, is not demarcated anteriorly from the larger region of cell division and growth just in front. An anterodorsally located anal region, as in certain tail regenerates, or in normal specimens of certain aquatic Glossocolecids, with an axial relationship simultaneously to a number of segments represented only by ventral rudiments, appears to have no more of the character of a segment. Even in old Lumbricid and Megascolecid worms, where the terminal region, superficially at least, may have much more of the appearance of a segment, setae, nephropores and pigment are also lacking, except when in an anterior portion a new segment is being differentiated. Finally, the strictly anal region, apparently in all species that have been studied (Hess, 1925; Imai, 1928; Ogawa, 1934), does not have a typical neuromere.

Is the anal region, in these circumstances, a segment? Rather does it not appear to be a perpetuation in the postembryonic period of an embryonic growth zone, morphologically rather undifferentiated but physiologically specialized mainly for continuation of growth, gradually decreasing in size with age, but even with greatest known reduction still retaining capacity for some slow production of new segments?

Actual rates of segment formation and the methods of segment formation have had little attention in the literature on growth in earthworms. From data above and in other publications several rates have been calculated or estimated. In *P. sansibaricus* segments may be formed, in the cocoon, at a rate of about five per diem for the entire embryonic period of 9-18 days. Similar or possibly somewhat higher rates may characterize *E. foetida* and *L. mauritii*. In *P. communissima,* which deposits in the fall cocoons that hatch in the spring (March-

April), rate of segment formation in embryonic growth would appear to be definitely lower, perhaps about 1 per diem or less (Ogawa, 1939). In tail regenerates of *P. excavatus* the rate at certain levels may be 3.8 + (or 1 + mm.) per diem for the first fourteen days (Gates, 1927, p. 359). In tail regenerates of *E. foetida* (Moment, 1946, p. 496), assuming a thirty-day regeneration period, the per diem rate of segment formation varied according to level as follows: at 40/41, 1.5-1.8; at 50/51, 0.7-1.8; at 60/61, 0.6-1.2; at 70/71, 0.2-1.0; at 80/81, 0.06-0.7. In homomorphic tail regenerates from small, two-surfaced substrates, in spite of seemingly unnoticed autotomy of some regenerated segments (Morgan, 1902, pp. 574-576) a rate of 1-3 per mensem in the third to seventh months apparently was obtained. *P communissima* forms new segments at a rate of about one a week from time of hatching in the spring until sexual maturity after which the worms die (Ogawa, 1939).

Externally recognizable morphological characteristics also permit recognition of different methods of segment formation.

One method is that characterizing the embryo to some unknown time prior to hatching and which may be referred to as embryonic.

A second method is that indicated by a dorsal anus, apparently enabling production of a number of segments almost simultaneously. This method may, pending further study, be designated in accordance with gradient of differentiation, ventro-dorsal or more conveniently ventral. It has a certain similarity to the embryonic method in that metameres are first demarcated ventrally, differentiation being continued laterally and finally dorsally. Although this method seems not to have had especial attention dorsal location of the anus in tail regenerates of Lumbricids has been known for more than fifty years, having been figured (usually without posterior lobe) by various continental authors. Most recent mention appears to have been that of Liebmann (1942a, p. 162) who noted that in tail regenerates of *E. foetida* at 50/51, the anus still opened dorsally at three weeks. Eventually the anus is moved posteriorly and finally becomes strictly terminal. In certain aquatic Glossocolecids the anus of the young tail regenerate is at first terminal but becomes dorsal and remains so permanently as in normal worms. As already indicated all of the Glossoscolecid species mentioned earlier have, relative to their size, unusually large numbers of segments.

A third method of segment formation is indicated by location of the anus at the very end of the body, on the posterior face, and may accordingly be called terminal (though anteroposterior more accurately indicates the differentiation gradient). This sort of growth zone may be large and because of its size, lack of pigmentation, etc., readily recognizable. In that case the appearance of the zone is such as to indicate production of several segments almost simultaneously. The zone may be reduced in size. So much so as to constitute only an unrecognized anterior portion of a small anal region previously regarded as a single segment. In that anterior portion there is usually recognizable only the rudiment of a single segment. With such marked reduction in size of the growth zone there presumably would be associated similar reduction in rate of growth. Thus a rapid and a slow method of terminal growth may be distinguished. The former has been found in just-hatched and early juvenile stages, occasionally to and even in sexual individuals (*P. sansibaricus, L. mauritii*), as well as in tail regenerates at certain stages and levels. The other has been found in adult-sized individuals (clitellate as well as pre- and postsexual aclitellate), juveniles, and tail regenerates at certain stages and levels.

Recognition of these different methods not only in normal but also in regenerative growth in the few species that were studied seems to indicate necessity for caution (hitherto unobserved) in generalization regarding growth in earthworms.

Presence, in certain species, of a growth zone in every available stage, indicates a theoretical possibility of continuous segment production through life. Whether segment formation is actually continued through sexual seasons and during periods of obligatory or facultative diapause remains to be determined. As a facultative diapause can be experimentally induced simply by allowing the media in which the worms are kept to dry that aspect of the problem would seem to be not too difficult to solve.

Summary

According to the evidence of growth regions and segmental counts, in the earthworms studied, postembryonic growth involves production of new segments. Methods of segment production and the role of segment formation in postembryonic growth, contrary to previous suppositions, are not uniform throughout the several families. In species

of certain Megascolecid genera the young hatch after a short embryonic period of 2-3 weeks with one-fourth to one-third of the maximal segment number for the species. In an early postembryonic period enough additional segments usually are produced to bring the total to a majority of the species maximum. Segment formation by the rapid terminal method may be continued until (after?) breeding but usually is replaced sometime before this by the slow terminal method. Other Megascolecid species may have a much longer embryonic period, of 4-5 months, and then hatch with a very considerable majority of the segmental complement, adding new segments only by the slow terminal method at rate of about one a week until maturity. In certain aquatic Glossoscolecids formation of segments is by a rapid ventral method. In tail regenerates segments may be formed by rapid ventral, rapid terminal or slow terminal methods.

Continuous segment formation throughout life, in case of the Glossoscolecidae always by the rapid ventral method, appears to be theoretically possible though effect of intervention of reproduction and diapause (facultative or obligatory) is unknown.

References

1. Gates, G. E. 1941. Further notes on regeneration in a tropical earthworm, *Perionyx excavatus* E. Perrier 1872. *J. Exp. Zool.* **88**, pp. 161-185.
2. ————. 1945. The earthworms of Allahabad. *Proc. Nat. Ac. Sci. India,* **15**, pp. 44-56.
3. Hess, W. N. 1925. Nervous system of the earthworm, *Lumbricus terrestris* L. *J. Morph.* **40**, pp. 235-259.
4. Hyman, L. H. 1940. Aspects of regeneration in annelids. *Am. Nat.* **74**, pp. 513-527.
5. Imai, T. 1928. Nervous system of the earthworm, *Perichaeta megascolidioides,* Goto and Hatai. *Sci. Reps. Tohoku Univ.* (*4*), **3**, pp. 443-460.
6. Korschelt, E. 1914. Über Transplantationsversuche, Ruhezustände und Lebensdauer der Lumbriciden. *Zool. Anz.* **4**, pp. 537-555.
7. Liebmann, E. 1942. The correlation between sexual reproduction and regeneration in a series of Oligochaeta. *J. Exp. Zool.* **91**, pp. 373-389.
8. ————. 1943. New light on regeneration of *Eisenia foetida* (Sav.). *J. Morph.* **73**, pp. 583-609.
9. Moment, G. B. 1946. A study of growth limitation in earthworms. *J. Exp. Zool.* **103**, pp. 487-506.
10. Morgan, T. H. 1902. Experimental studies of the internal factors of regeneration in the earthworm. *Arch. Entwickmech. Org.* **14**, pp. 562-591.
11. Ogawa, F. 1934. The number of ganglion cells and nerve fibres in the nervous system of the earthworm, *Pheretima communissima. Sci. Reps. Tohoku Univ.* (*4*), **8**, pp. 345-368.
12. ————. 1939. The nervous system of earthworm (*Pheretima communissima*) in different ages. *Sci. Reps. Tohoku Univ.* (*4*), **13**, pp. 395-488.
13. Sun, K. H., Pratt, K. V. 1931. Do earthworms grow by adding segments? *Am. Nat.* **65**, pp. 31-48.

Growth, 1948, **12**, 181-202.

EFFECTS OF THYROID INHIBITORS UPON TAIL REGENERATION IN THE TADPOLE[1]

MOTHER M. BONAVENTURE GHIDONI, O.S.U.

Department of Biology, The Catholic University of America, Washington, D. C.
(Received for publication March 26, 1948)

A. INTRODUCTION

The rapidity and completeness with which regeneration of the tail occurs in amphibian larvae has made these animals favored objects for the investigation of regenerative processes. Zeleny (16) carried out a detailed study of tail regeneration in *Rana clamitans* tadpoles and distinguished three successive phases in the process. Immediately following amputation there is a period of "regulation" characterized by cell migration and reorganization at the cut surface. Following this comes a phase of proliferation during which the regenerating area rapidly increases in size. This is succeeded by a period of cell and tissue differentiation which is accompanied by a gradual decline in the rate of growth of the regenerating region. The time required for these phases, other factors being constant, varies with the extent of the amputation. When one-half of the tail is removed the regulative phase occupies two to four days, the proliferative phase lasts three to six days and the differentiative phase continues throughout the rest of the regenerative period.

Speidel (29) found that the course of regeneration is definitely affected by treatment with thyroid extract and, moreover, that the effect differs depending upon the phase of regeneration during which the treatment is instituted. If thyroid administration is begun shortly before or coincident with amputation of the tail, the regulative phase

[1]A contribution from the Department of Biology, The Catholic University of America, Washington, D. C. This paper is based on the author's dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The writer wishes to express her deep appreciation to Professor William Gardner Lynn, who suggested this problem to her, for his many helpful suggestions both during the course of the investigation and in the preparation of the manuscript. To Professor Edward G. Reinhard and Reverend Father Henry Wachowski sincere thanks are due for their helpful suggestions.

is interfered with and almost no regeneration occurs. If, on the other hand, administration of the extract is deferred until near the end of the regulative phase or later, growth and proliferation are accelerated and the onset of the differentiative phase is hastened. Thus in the first case the hyperthyroid condition results in inhibition of regeneration, while in the second it causes an increase in the growth rate and a rapid maturing of the tissues. Speidel's experiments with tadpoles of the wood frog (*Rana sylvatica*), the bullfrog (*Rana catesbiana*), the tree frog (*Hyla crucifer*), and the toad (*Bufo americanus*) showed that the general effect of thyroid extract upon regeneration is similar in all of these forms.

In view of these results it would be of interest to follow the course of regeneration in tadpoles in which the thyroid gland is absent or non-functional. A search of the literature reveals almost no information on this subject. There appears to be no comprehensive study of regeneration in thyroidectomized or hypophysectomized animals despite the fact that the techniques for performing these operations in amphibian larvae are well known and quite simple.

The present investigation is an attempt to approach this problem, not by operative techniques, but by use of some of the newly developed chemical inhibitors of the thyroid. The independent discovery by Richter and Clisby (42), Kennedy (42), and Mackenzie and Mackenzie (43) of the goiterogenic activity of the thioureas has led to extensive study of these drugs. It has been shown that in mammals administration of these substances causes hypertrophy of the thyroid epithelium and reduction in the amount of colloid with accompanying symptoms of hypothyroidism.

Gordon, Goldsmith, and Charipper (43, 45) were the first to report experiments dealing with the effects of thiourea upon the amphibian larva. They found that *Rana pipiens* tadpoles raised in a .033 per cent solution of the drug from the stage of first appearance of the hind-limb buds failed to metamorphose, having hind limbs only three to five millimeters long at the time when the controls had completely transformed into frogs. Hughes and Astwood (44) obtained similar results using a related compound, thiouracil, in a concentration of 1:2000. Since simultaneous administration of thyroxin to these animals promptly induced metamorphosis it seems clear that the thiouracil does not act directly upon the tissues but brings about its

effect through interference with the normal production of the thyroid hormone. Still another compound, phenylthiourea, in a concentration of .005 per cent has been found by Lynn (47) to be an effective inhibitor of the thyroid function in the anuran *Eleutherodactylus ricordii*.

In the present study two of these drugs, thiourea and phenylthiourea, have been used as thyroid inhibitors and tail regeneration under various conditions of treatment has been investigated.

B. Materials and Methods

Tadpoles of the bullfrog (*Rana catesbiana*) were obtained from a pond at McDonogh, Maryland. Large numbers were available, making it possible to select specimens of uniform size. Tadpoles of the leopard frog (*Rana pipiens*) were obtained by pituitary-induced ovulation and artificial insemination using adults obtained from a dealer in New York State. For each experiment tadpoles from a single clutch of eggs were used and were carefully selected for uniformity of size.

The bullfrog tadpoles were kept individually in glass jars each containing one liter of tap water. The only exception to this was in the first experiment to be reported, in which five tadpoles were kept in each jar. The leopard frog larvae were raised in white enameled pans measuring 9 x 15 x 2 inches. Each pan contained 10 tadpoles in one liter of culture medium. Animals were fed principally upon pabulum and strained liver-soup preparation. All cultures had excess food available and uniform conditions of light and temperature were maintained. The culture medium was changed daily except in cases specifically noted in the accounts of individual experiments.

Tail amputation was carried out under anesthesia with MS-222 in concentration of 1:4500. In all experiments the distal two-thirds of the tail was removed. Measurements of body dimensions and length of regenerating tail were taken by means of dividers under a binocular microscope. At various stages in the course of the experiments, tadpoles were preserved in 10 per cent formalin for histological study. Materials for sectioning were dehydrated in cellosolve, cleared in xylol, embedded in paraffin, sectioned at 10 u and stained with Mallory's triple stain or Harris' hematoxylin and eosin.

C. Results

1. Effects of thiourea upon regeneration when administered during the proliferative phase.

Speidel's (29) experiments demonstrated that the accelerating effect of thyroid substance upon tail regeneration is most pronounced when treatment is instituted during the proliferative phase. It therefore seemed best to begin the present investigation with a study of the results of thyroid inhibition during this phase. This experiment also served as an exploratory investigation of the effects of various concentrations of thiourea and the relative effectiveness of administration by injection, as compared with administration by solution of the drug in the culture medium.

Thirty-five bullfrog tadpoles ranging from 68 to 72 mm. in total length were divided into seven groups of five. Each group was kept in a large glass container holding two liters of culture medium. The distal two-thirds of the tail was removed from all animals on the same day. Regeneration was allowed to proceed without treatment for six days at which time the process was in the early proliferative phase. The various groups were then subjected to different treatment as follows:

Group 1: given daily intra-peritoneal injections of .1 cc of 0.1 per cent thiourea
Group 2: given daily intra-peritoneal injections of .1 cc of 0.05 per cent thiourea
Group 3: given daily intra-peritoneal injections of .1 cc of 0.005 per cent thiourea
Group 4: kept in culture medium consisting of a solution of .05 per cent thiourea in tap water.
Group 5: kept in culture medium consisting of a solution of .01 per cent thiourea in tap water.
Group 6: controls, given daily injections of .1 cc of distilled water.
Group 7: controls, kept in tap water.

Culture media were changed and injections were given daily for a period of 27 days. Measurements of the regenerating tails were made daily and the results are given in the accompanying table and graph (table 1 and figure 1). Since the individual measurements within a group never varied more than three millimeters, only the averages are given here. Also, since both groups of controls ran exactly parallel,

TABLE 1

EFFECTS OF THIOUREA UPON TAIL REGENERATION, WHEN ADMINISTERED DURING THE PROLIFERATIVE PHASE

Day of Regeneration	Average length of regenerating tail in mm. Thiourea injections 0.1%	0.05%	0.005%	Thiourea solutions 0.05%	0.01%	Controls
6	2	2	2	2	2	2
7	2	2	2	2	2	2
8	2	3	3	3	2	2
9	3	3	3	3	3	3
10	3	4	4	4	4	4
11	4	4	5	4	5	5
12	5	5	6	4	6	6
13	6	6	7	5	7	7
14	6	6	7	5	8	7
15	7	6	7	6	8	8
16	7	6	7	7	8	9
17	7	6	7	7	9	10
18	7	7	7	8	9	10
19	7	7	7	8	10	11
20	8	8	7	8	11	11
21	8	8	8	8	11	12
22	8	8	8	8	11	12
23	9	8	8	9	12	12
24	9	8	9	9	13	13
25	9	8	10	9	14	14
26	10	8	10	9	14	14
27	10	8	10	9	14	15
28	10	9	10	9	15	15
29	10	9	10	9	15	15
30	10	9	10	10	15	15
31	11	9	11	11	16	15
32	11	9	11	11	16	15

averages are given for the two groups. It must be mentioned that during the course of the experiment four animals were lost. Two died in the .05 per cent solution; one on the seventh day of treatment and one on the twenty-fourth day. Two died in the group injected with .1 per cent thiourea; one on the thirteenth day and one on the seventeenth day. No other animals showed any signs of ill effects throughout the experiment.

Examination of the graph shows clearly that treatment with thiourea causes a definite decrease in the rate of tail regeneration. All three of the groups in which administration was by injection showed a pronounced effect which first became apparent about nine days after the beginning of treatment (fifteenth day of regeneration). The strength of the dosage, within the limits tested, seems to bear no

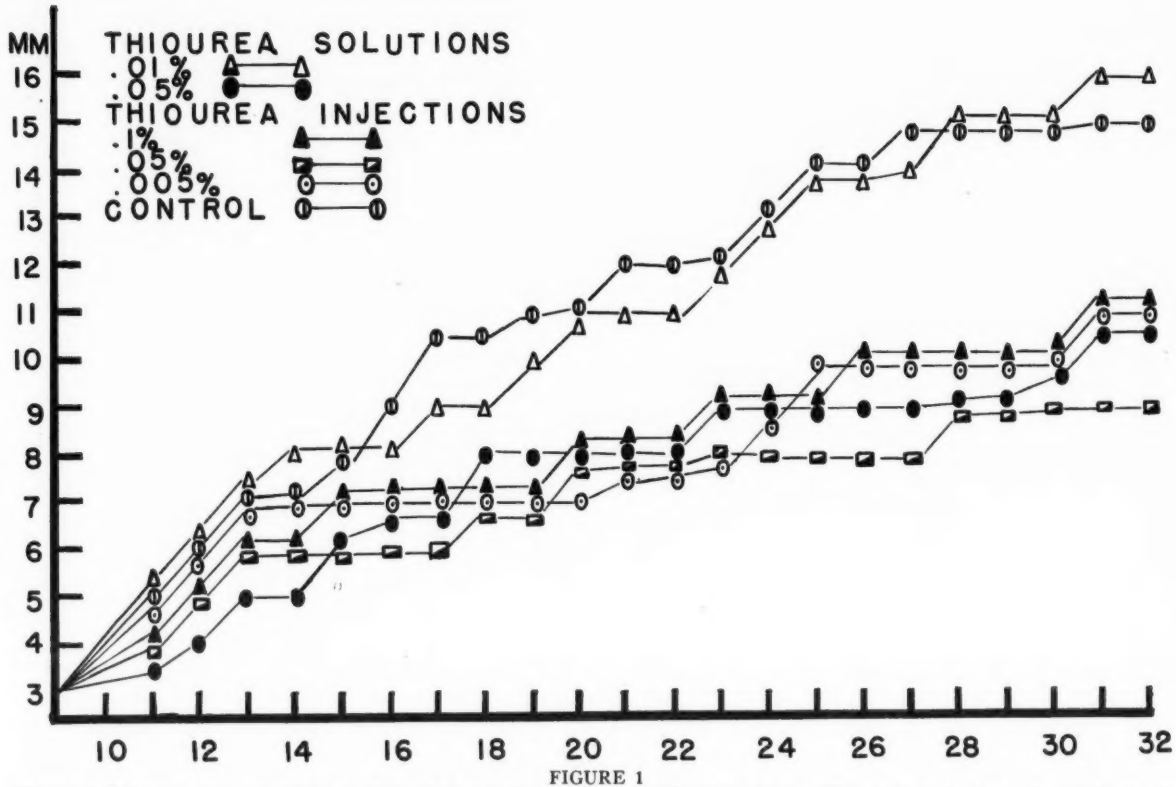


FIGURE 1

Effects of Thiourea upon regeneration when administered during the proliferative phase. (The abscissa is days of experiment, the ordinate is average length of regenerated tail in millimeters.)

relation to the intensity of the effect. This is what would be expected if all three dosage levels are sufficient to cause complete thyroid inhibition, and if the effect of the drug is assumed to be due to its influence on thyroxin-production rather than to any direct action on the regenerating tissues.

In the case of administration by addition of thiourea to the culture medium the situation is somewhat different. The curve for regeneration in .05 per cent thiourea shows an effect which parallels that produced by the various injection levels. On the other hand, the curve for regeneration in .01 per cent thiourea is not significantly different from that for the controls. This indicates that the latter concentration is too low to inhibit thyroid activity when administered in the culture medium and that the thyroxin level in tadpoles raised in this solution remains high enough to permit regeneration at the normal rate.

2. Effects of thiourea upon regeneration when administered prior to, or coincident with, tail amputation.

In view of Speidel's findings that thyroxin inhibits regeneration if administration is begun before or simultaneously with tail amputation, it now seemed desirable to study the course of regeneration in tadpoles in which thiourea treatment was instituted prior to amputation. Under such treatment the animals would presumably have a very low thyroxin level from the very beginning of the regeneration period. The experiment just reported indicates that an effective method of administration of the drug is injection at a concentration of .05 per cent and this method was therefore used exclusively in the present series.

Thirty-six bullfrog tadpoles ranging from 68 to 75 mm. in length were used. Because of the somewhat greater range in length as compared with the previous group it was decided to keep individual controls for each experimental animal. Eighteen pairs were therefore selected so that the two members of a pair differed by not more than two millimeters in total body length. One animal of each pair was given the injections while the other served as its control. Treatment was as follows:

Group A: Seven animals were given injections of .2 cc of .05 per cent thiourea on alternate days for 24 days. At the end of this time tail amputations were performed on these tadpoles

and their controls. The injections were continued throughout the course of the experiment and measurements were taken every other day from the fourth to the sixteenth day of regeneration.

Group B: Seven experimental animals were given injections of .2 cc of .05 per cent thiourea on alternate days for 16 days before amputation. Injections were continued and measurements taken throughout the first 16 days of regeneration.

Group C: Four experimental animals received their first injection on the same day that the tails were amputated. Injections were continued on alternate days for the first 16 days of regeneration as in the other groups.

The individual measurements of the regenerating tails are given for all of these animals and their controls in table 2. It will be seen that none of these treatments had any significant effect upon the early stages of regeneration. The regulative phase is neither retarded nor accelerated, experimental animals and controls regenerating at the same rate throughout the first 10 days of regeneration. After this a slowing down of the growth-rate of the tails of the injected tadpoles is shown in most of the pairs. This was, of course, to be expected since the injections were being continued and it has already been shown that treatments during the proliferative phase result in retardation of the regenerative rate. In the present series of experiments this effect is first apparent in most pairs on the 14th day of regeneration, which agrees closely with the time required for the effect to manifest itself in the first series reported.

3. Effects of thiourea and phenylthiourea upon tail regeneration during different periods of the larval history.

Zeleny (16) has noted that the normal rate of regeneration varies with the age of the larva. Since this can probably be attributed in part to the normal increase in activity of the thyroid gland in older tadpoles which are approaching metamorphosis, an experiment was now undertaken to test the effects of thyroid-inhibition upon regeneration in young and old larvae. *Rana pipiens* tadpoles were used for this study because their short larval period makes them more suitable than the bullfrog for such work. Since it was also desired to test the relative effects of thiourea and phenylthiourea, both of these

TABLE 2
EFFECTS OF THIOUREA UPON REGENERATION WHEN ADMINISTERED PRIOR TO, OR COINCIDENT WITH, TAIL AMPUTATION

Group	Animal	Days of Regeneration 4	6	8	10	12	14	16
	5 C	1	2	3	5	7	9	10
	5 I	1	2	3	5	7	8	9
	6 C	1	2	3	5	7	10	10
	6 I	1	2	3	4	6	7	8
	4 C	1	2	3	4	7	9	9
A	4 I	1	2	3	5	7	8	8
	9 C	1	2	4	5	6	8	9
	9 I	1	2	4	5	6	6	7
	2 C	1	2	3	5	7	9	10
	2 I	1	2	3	4	6	9	10
	16 C	1	2	3	4	7	8	9
	16 I	1	2	3	5	6	7	8
	18 C	1	2	3	5	7	9	9
	18 I	1	2	3	4	5	6	6
	15 C	1	2	3	5	6	7	9
	15 I	1	2	3	4	5	6	8
	1 C	1	2	4	6	8	9	10
	1 I	1	2	4	6	8	10	11
	3 C	1	2	3	5	7	7	9
	3 I	1	2	3	4	5	6	8
B	12 C	1	2	4	4	7	8	10
	12 I	1	2	4	4	6	7	9
	10 C	1	2	4	5	6	7	8
	10 I	1	2	5	6	8	9	10
	13 C	1	2	3	5	7	8	9
	13 I	1	2	3	5	6	7	9
	14 C	1	2	3	5	6	7	8
	14 I	1	2	3	4	6	7	9
	7 C	1	2	3	5	7	10	10
	7 I	1	2	3	4	6	8	8
	8 C	1	2	4	5	6	8	9
	8 I	1	2	4	4	6	7	8
C	11 C	1	2	4	5	7	8	10
	11 I	1	2	4	4	6	9	10
	17 C	1	2	3	5	7	9	9
	17 I	1	2	3	4	6	8	9

C—control animal.
I—injected animal. Regenerated tail length in mm.

drugs were utilized. Phenylthiourea had already been found to be effective in inhibiting thyroid function when administered in the culture medium in the rather low concentration of .005 per cent. In view of this fact, and in consideration of the small size of *Rana pipiens* larvae, it was thought that thiourea might also be effective at

this concentration rather than at the higher ones which had proved necessary in the bullfrog. In this experiment, therefore, both drugs were administered by being added to the culture media in a concentration of .005 per cent.

Tadpoles hatched in the laboratory were raised in enamel pans, ten animals in each pan, until they reached the desired stages. The following experimental series were run:

Group A: Twelve cultures of larvae (120 animals) were raised in tap water until the tadpoles reached an average length of 24 mm. At this time they were 53 days of age. Four of the cultures were then transferred to .005 per cent thiourea, four were transferred to .005 per cent phenylthiourea and four were continued in tap water as controls. Amputation of the tail was carried out in all animals and the course of regeneration was followed by means of measurements made at varying intervals.

Group B: Twelve cultures were raised until the tadpoles reached an average length of 34 mm. (81 days). At this time the larvae all had well developed hind-limb buds 2 to 3 mm. long. Four cultures were then transferred to .005 per cent thiourea, four to .005 per cent phenylthiourea and four were used as controls. Tail amputation was performed and regeneration was followed as before.

TABLE 3

EFFECTS OF .005% THIOUREA AND .005% PHENYLTHIOUREA UPON TAIL REGENERATION IN YOUNG AND OLDER LARVAE

	Day of Regeneration	Average length of regenerating tail in mm. Thiourea	Phenylthiourea	Controls
Group A	8	3	3	3
	10	3	3	4
	13	5	3	6
	19	9	5	9
	26	13	7	12
	38	15	10	14
Group B	7	3	2	3
	14	8	6	7
	21	11	7	10

The results of this experiment are given in table 3. It will be seen that in both Group A and Group B the animals raised in .005 per cent thiourea regenerated at essentially the same rate as did the controls.

Clearly this concentration is not high enough to be effective when added to the culture medium. On the other hand, .005 per cent phenylthiourea caused a retardation in both young and old larvae, the effect being first shown during the second week of regeneration. The primary purpose of this experiment was to ascertain whether there is any difference in the effect at early and late stages. There is no evidence that any such difference exists. It is to be noted, however, that the regeneration rates in the controls of Groups A and B did not differ significantly either, a fact which is not in agreement with previous observations on the relation between age and rate of tail regeneration.

To illustrate the results of this experiment, specimens from Group B were photographed and are shown in figures 2, 3, and 4. Figure 2 shows tadpoles on the seventh day of regeneration. The uppermost animal is a control, the middle animal is one treated with .005 per cent phenylthiourea and the lower specimen is from a .005 per cent thiourea culture. The regenerated portions of the tails of the control and thiourea-treated tadpoles both measure 3 mm., that of the phenylthiourea-treated animal measures 2 mm. Figure 3 shows specimens from the same three cultures on the fourteenth day of regeneration. The animals are arranged in the same order as in the preceding figure. The regenerated regions of the tails of control and thiourea-treated specimens now measure 8 mm.; that of the phenylthiourea-treated animal measures 6 mm. Figure 4 shows specimens on the twenty-first day, the tail measurements at this time being 13 mm. for the control and thiourea-treated tadpoles and 8 mm. for the phenylthiourea-treated specimens.

4. Effects of thiourea upon tail regeneration after repeated amputation.

The animals from Group A in the preceding experiment which had been operated upon at the 24 mm. stage had well-formed tails after 38 days of regeneration. It was decided that they could again be utilized, and all were therefore subjected to a second amputation. This second cut was made just proximal to the level of the first. At this time the animals were 91 days old. The control tadpoles and those in .005 per cent thiourea were beginning to show signs of the onset of metamorphosis, the hind limbs having reached an average

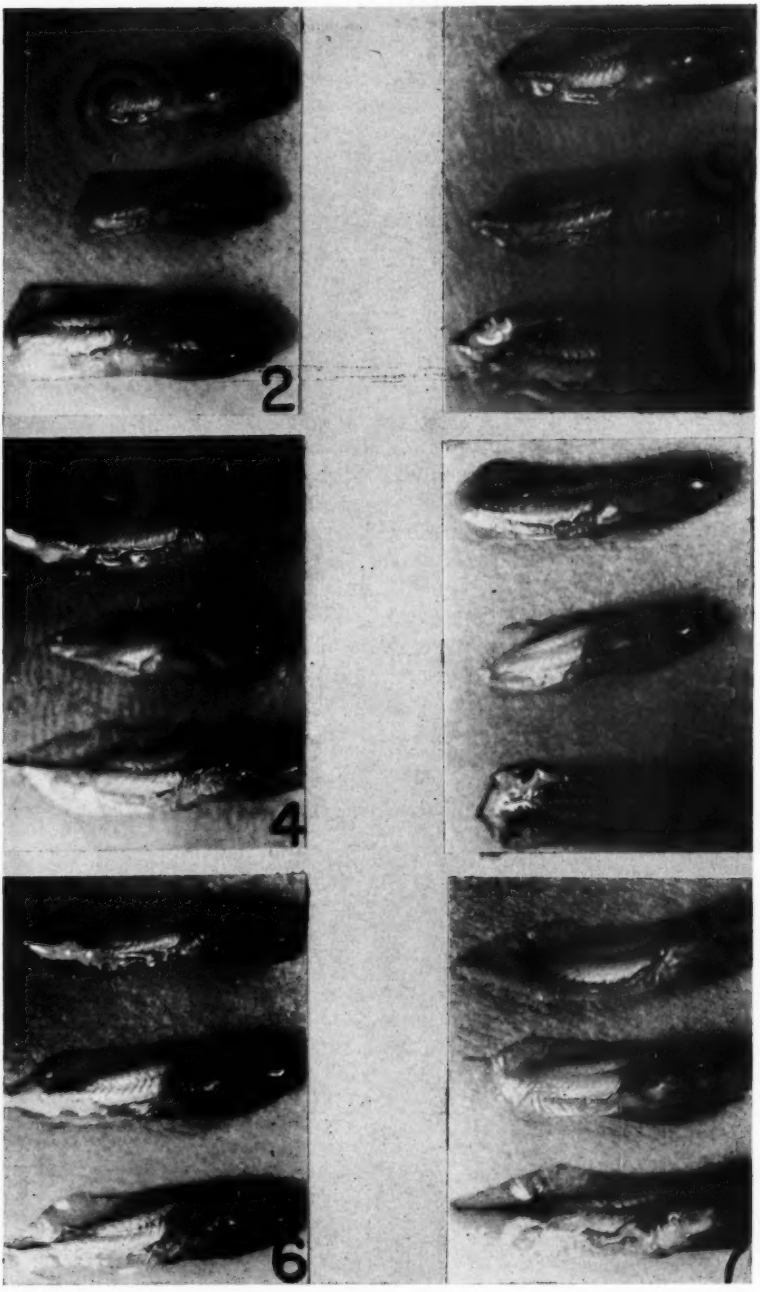

2
4
6
7

PLATE I

(Figures 2-7)

Photographs of experimental and control *Rana pipiens* tadpoles. x 1. In each figure the uppermost animal is the control, the middle animal was raised in .005 per cent phenylthiourea, and the lower animal was raised in .005 per cent thiourea.

2. Animals from Series 3, Group B on the 7th day of regeneration.
3. Animals from Series 3, Group B on the 14th day of regeneration.
4. Animals from Series 3, Group B on the 21st day of regeneration.
5. Animals from Series 4 on the 7th day of regeneration.
6. Animals from Series 4 on the 14th day of regeneration.
7. Animals from Series 4 on the 21st day of regeneration.

length of 6 mm. The tadpoles in .005 per cent phenylthiourea had hind limbs only 2 mm. long. Measurements of the regenerating tails of the tadpoles of Group A after the second amputation are given in

TABLE 4
EFFECTS OF .005% THIOUREA AND .005% PHENYLTHIOUREA UPON TAIL REGENERATION AFTER SECOND AMPUTATION

Day of Regeneration	Average length of regenerating tail in mm.		
	Thiourea	Phenylthiourea	Controls
7	4	3	4
14	9	7	8
21	13	9	12
28	16	12	15

table 4 and specimens on the seventh, fourteenth, and twenty-first days of regeneration are illustrated in figures 5, 6, and 7. Again the animals in .005 per cent thiourea paralleled the controls while the tadpoles treated with .005 per cent phenylthiourea showed a retardation of regeneration rate. An unexpected result is to be observed by comparison of the figures in table 4 with those given for the correspondingly treated animals in table 3. It appears that in both control and experimental animals regeneration after a second amputation proceeds at a somewhat faster rate than does regeneration after first amputation.

In an attempt to obtain confirmation of this observation a further experiment was now performed. Thiourea alone was used but the concentration was increased to .033 per cent. The work of Gordon, Goldsmith, and Charipper (43) has shown that this concentration is sufficient to cause failure of metamorphosis in *Rana pipiens*.

Twenty tadpoles hatched in the laboratory were raised for 23 days. They were then placed in .033 per cent thiourea and maintained in this solution throughout the rest of the experiment. After 11 days treatment the first amputation was performed. The tadpoles averaged 25 mm. in length at this time. A second amputation was made 18 days after the first when the tadpoles were 52 days old and averaged 40 mm. in length. The third amputation was made 17 days later, the animals then being 69 days old. The average length at the time of the third amputation was only 42 mm., body growth having practically ceased at about 55 days.

Measurements of the regenerating tails were taken on alternate

days throughout the experiment. At the close of the experimental period the tadpoles were 86 days old and had been kept in .033 per cent thiourea for 63 days. None of them showed any signs of hind-limb growth. Many control animals from the same egg mass had completely metamorphosed by this time.

TABLE 5
EFFECTS OF THIOUREA UPON TAIL REGENERATION AFTER REPEATED AMPUTATIONS

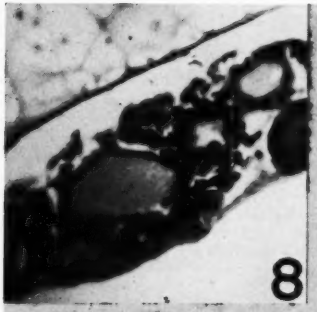
Day of Regeneration	Average length of regenerated tail in mm.		
	1st amputation	2nd amputation	3rd amputation
4	1	2	3
6	3	4	5
8	4	6	6
10	7	8	7
12	8	9	9
14	10	10	10
16	11	10	10

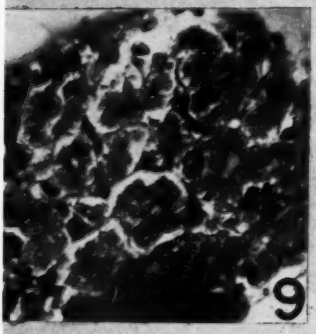
The results of this experiment are given in table 5. It will be noted that the rate of growth of the tail is directly correlated with the number of amputations, being slowest after the first amputation and fastest after the third during the first week of regeneration. After the tenth day, however, the growth rates in all three groups run closely parallel.

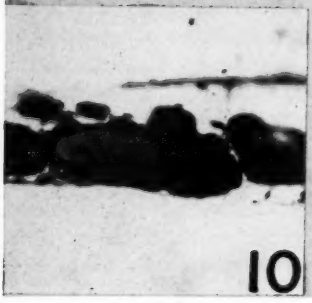
5. Histological observations.

At intervals during the course of the various experiments described in this paper some individuals from each series were preserved. Later, selected specimens were sectioned to permit histological examination of their thyroids. This makes it possible to correlate the influence of the drugs upon tail regeneration with their effects upon the thyroid gland. As expected, it was found that animals which had received thiourea in such low concentrations that no effect upon regeneration could be detected had thyroids which were nearly normal in appearance. On the other hand, all animals whose regeneration had been definitely retarded by treatment with the drugs showed markedly affected thyroids.

The three photomicrographs shown in figures 8, 9, and 10 will suffice to illustrate these observations. The thyroid glands shown here are those of *Rana pipens* tadpoles used for the study outlined in section 3. Figure 8 shows the thyroid of an animal which had been


8


9


10

12

PLATE II

(Figures 8-12)

8. Section of thyroid gland of larva raised in .005 per cent thiourea solution for 75 days. x240.

9. Section of thyroid gland of control larva. x240.

10. Section of thyroid gland of larva raised in .005 per cent phenylthiourea for 75 days. x240.

11. Longitudinal section of the regenerating tail of a control larva on the 21st day of regeneration. x240.

12. Longitudinal section of the regenerating tail of a larva treated with .005 per cent phenylthiourea. Twenty-first day of regeneration. x240.

in .005 per cent thiourea for 75 days. Figure 9 shows the thyroid of an animal of the same age which had been in .005 per cent phenylthiourea for 75 days. Figure 10 shows the thyroid of a control of the same age. Obviously the phenylthiourea has caused a marked hypertrophy of the gland and an almost complete loss of colloid. The hyperemic condition of this thyroid is revealed by the many blood corpuscles scattered about between the follicles. The gland of the thiourea-treated tadpole differs but little from that of the control. There seems to be evidence of some increase in vascularity, but the follicular epithelium remains low and the amount of stored colloid present lies in the normal range. It will be remembered that the animals treated with .005 per cent thiourea in this experiment paralleled the controls in regeneration rate while those treated with .005 per cent phenylthiourea showed a retardation.

Some observations have also been made upon the histological features of the regenerating tail in experimental and control larvae. The general course of histo-differentiation in the control animals follows that described by previous authors (Allen 11, Naville 22). In experimental animals in which the regeneration has been markedly retarded there is good evidence that the retardation involves the process of differentiation as well as the rate of growth. Comparison of the regenerating tails of experimental animals with those of controls at different stages, shows that the time of appearance of myofibrillae and of notochord tissue is considerably later in the former than in the latter. This difference is illustrated in figures 11 and 12, which show longitudinal sections of the tails of *Rana pipiens* larvae on the twenty-first day of regeneration. The control animal (fig. 11) has well-differentiated muscles in the regenerated area while the experimental animal (fig. 12) has only a few myofibrillae at the proximal end of the new tail. Further study of this phase of the problem is needed, but it seems clear that thyroid-inhibition does affect the rate of differentiation in regenerating tissues.

D. Discussion

The role of the thyroid secretion in anuran metamorphosis has been widely studied since the days of Gudernatsch's (12) pioneering experiments. It has been repeatedly demonstrated that removal of the thyroid anlage from the young larva results in complete failure to

metamorphose, while administration of the thyroid hormone to early tadpoles causes them to transform into frogs precociously. The phenomenon of metamorphosis is a complex one involving rapid growth and differentiation of some structures, such as the limbs, and resorption and ultimate disappearance of others, such as the gills and tail. It has been shown, however, that all of these changes are induced by the thyroid hormone, the differences in response in different tissues being due to the diverse constitutions of the tissues themselves.

Since the processes of growth and differentiation which occur during regeneration are in many respects similar to those of embryonic development, it was early suspected that they too might be affected by the thyroid secretion. Allen (18), in a brief abstract, recorded experiments upon tail regeneration in *Rana pipiens* tadpoles some of which had been thyroidectomized while others were fed thyroid extracts. He reported that in both of these groups, regeneration proceeded at the same rate as in untreated controls, and he therefore concluded that the thyroid gland does not influence the process of regeneration. As has been pointed out, however, Speidel's (29) very detailed experiments later demonstrated quite clearly that tail regeneration is affected by thyroid administration. Treatment during the early stages of regeneration causes inhibition of growth and differentiation; treatment during the later phases stimulates these processes.

In the present study the normal activity of the thyroid gland was reduced by administration of thyroid-inhibiting drugs to regenerating animals. It was found that if such inhibition is exerted during the early proliferative phase of regeneration the rate of growth of the regenerating tissue is definitely retarded. This is in complete agreement with Speidel's findings. On the other hand, treatment with thyroid inhibitors begun prior to or at the start of regeneration had no effect upon the early regenerative processes. Since Speidel had found that thyroxin treatment at this time caused a definite inhibition of regeneration, it might have been expected that decreasing the thyroxin level would give the opposite effect, namely acceleration of the regeneration rate. Since this is not the case it seems logical to conclude that the early phase of regeneration normally goes on independently of the thyroid gland but that it may be adversely affected by abnormally high thyroxin levels. If this hypothesis is correct, one would expect no effect upon this phase when the thyroid gland is removed or inhibited.

Zeleny (16) has reported that the rate of regeneration is somewhat faster in younger larvae than it is in older larvae. In the present study an experiment to test the effects of thyroid inhibition upon regeneration in *Rana pipiens* tadpoles at early and late larval stages failed to show any difference in regeneration rate at these times. It must, therefore, be concluded that the effect of thyroid hormone upon regeneration does not differ at different ages, at least within the limits tested. It is possible that experiments covering a wider interval and extending into actual metamorphic stages might reveal some differences in the effect.

Hoskins and Hoskins (19) in the course of a comprehensive study of the growth of various organs in thyroidectomized tadpoles of *Rana sylvatica* made incidental observations upon regeneration of the tail in a single thyroidectomized larva which was subjected to four successive amputations. They reported that the time required to regenerate the lost tissues increased steadily with each amputation. On the other hand, Zeleny (16) maintains that in normal tadpoles the regeneration rate is faster after a second amputation than it is after a first. In the present series of experiments, preliminary observations on tadpoles subjected to a second amputation seemed to indicate that regeneration does occur more rapidly under this condition, in both experimental and control animals. A further study of this matter was then carried out with thiourea-treated animals subjected to three successive tail amputations. The results show that, even under conditions of thyroid inhibition, the regeneration rate does increase after repeated amputations. The increase in rate is slight, however, and is limited to about the first week of regeneration. After this time the rate levels off in all series.

Histological study of the thyroid glands of the animals used in these experiments has demonstrated that in all cases in which a definite effect upon tail regeneration was obtained as a result of the treatment, a marked hypertrophy and diminution of colloid had been produced in the thyroid. In cases in which the treatment had been ineffective, the thyroids showed little or no difference from those of control tadpoles. This evidence thus confirms the hypothesis that the effects of thiourea and phenylthiourea upon tail regeneration result from their influence upon the activity of the thyroid gland rather than from any direct effect upon the regenerating tissues.

E. Summary

1. Administration of thiourea, in proper dosage, to tadpoles of *Rana catesbiana* and *Rana pipiens* during the proliferative phase of tail regeneration results in a retardation of the rate of growth of the new tail.

2. The dosage required depends upon the method of administration. Daily injections of .1 cc of thiourea in concentrations as low as .005 per cent were found to be effective in the bullfrog. In the case of administration by solution of the drug in the culture medium, a .05 per cent solution was effective but a .01 per cent solution was ineffective.

3. Administration of thiourea prior to or coincident with tail amputation does not affect the rate of regeneration during the regulative phase.

4. Phenylthiourea causes a retardation of tail regeneration during the proliferative phase in *Rana pipiens* tadpoles when administered by solution in the culture medium in a concentration of .005 per cent.

5. The regeneration rate in young (53 day) and older (81 day) larvae of *Rana pipiens* is affected in the same way and to the same degree by administration of phenylthiourea. The possibility remains, however, that study of larvae of more widely different ages might reveal some differences in response.

6. When *Rana pipiens* tadpoles are subjected to several successive tail amputations the regeneration rate during the early phases of the process is slightly greater after the later amputations than it is after the first. This is true even in tadpoles which are under continuous treatment with thyroid-inhibitors.

7. Histological study of the thyroids of experimental and control animals indicates that the effect of thiourea and phenylthiourea upon tail regeneration is attributable to their influence upon thyroid activity and not to any direct effect upon the regenerating tissues.

8. Preliminary examination of sections of the tails of experimental and control animals indicates that the reduction of thyroxin level in the treated tadpoles not only causes a retardation in growth rate but also has an inhibiting effect upon the differentiation of muscle and notochordal tissue in the regenerating tail. Full confirmation of this observation must, however, await further study.

References

1. Allen, B. M. 1918. The relation of the thyroid gland to regeneration in *Rana pipiens*. *Anat. Rec.*, **14**, 85.
2. Allen, W. E. 1911. A study of the relation of tissue differentiation to the rate of growth during regeneration. *Biol. Bull.*, **21**, 187-206.
3. Gordon, A. S., Goldsmith, E. D., & Charipper, H. A. 1943. The effect of thiourea on the development of the amphibian. *Nature*, **152**, 504-505.
4. ———————. 1945. The effects of thiourea on amphibian development with a note on the ineffectiveness of sulfadiazine and para-aminobenzoic acid. Growth, **9**, 19-41.
5. Gudernatsch, J. F. 1912. Feeding experiments on tadpoles. I. Influence of specific organs given as food on growth and differentiation. *Arch. Entwmech. Org.*, **35**, 457-483.
6. Hoskins, E. R., & Hoskins, M. M. 1919. Growth and development of amphibia as effected by thyroidectomy. *J. Exper. Zool.*, **29**, 1-69.
7. Hughes, A. M., & Astwood, E. B. 1944. Inhibition of metamorphosis in tadpoles by thiouracil. *Endocrinology*, **34**, 138-139.
8. Kennedy, T. H. 1942. Thioureas as goitrogenic substances. *Nature*, **150**, 233-234.
9. Lynn, W. G. 1947. Effects of thiourea and phenylthiourea upon the development of Eleutherodactylus ricordii. *Anat. Rec.* **99**, 106.
10. Mackenzie, C. G., & Mackenzie, J. B. 1943. Effects of sulfonamides and thioureas on the thyroid gland and basal metabolism. *Endocrinology*, **32**, 185-209.
11. Naville, A. 1922. Histogénèse et régénération du muscle chez les Anoures. *Archiv. de Biol.*, **32**, 37-171.
12. Richter, C. P., & Clisby, K. H. 1942. Toxic effects of bitter tasting phenylthiocarbamide. *Arch. Path.*, **33**, 46-57.
13. Speidel, C. C. 1929. Studies of hyperthyroidism. VI. Regeneration phenomena in thyroid-treated amphibian larvae. *Amer. Jour. Anat.*, **43**, 103-165.
14. Zeleny, C. 1916. Studies on the factors controlling the rate of regeneration. *Illinois Biological Monographs*, **3**, 7-139.

Growth, 1948, **12**, 203-242.

THE EFFECTS OF ROENTGEN RAYS ON THE EMBRYOS AND LARVAE OF THE CHINOOK SALMON*

ARTHUR D. WELANDER, LAUREN R. DONALDSON, RICHARD F. FOSTER
KELSHAW BONHAM, AND ALLYN H. SEYMOUR

Applied Fisheries Laboratory, University of Washington, Seattle
(Received for publication April 15, 1948)

The effects of X-rays on the embryo and larva of the chinook salmon *Oncorhynchus tschawytscha* (Walbaum) were studied and are here summarized. In this evaluation an analysis was made of the effects of different dosages on gross pathology, mortality, growth in weight and length, development of various structures, and histopathology of various organs. A somewhat similar experiment on chinook salmon fingerlings has been reported by Bonham, et al (1947).

Materials and Methods

The chinook eggs used were the progeny of several parents artificially spawned at the Soos Creek Hatchery, State Fisheries Department, Auburn, Washington. The eggs had been mixed by routine handling at the hatchery and were in an early stage of development when received. This corresponded to the "eyed-egg" stage in which the pigment of the eye is visible through the shell.

The eggs were divided into seven lots and six of the lots were irradiated during the eyed stages with single doses of 250, 500, 1,000, 2,500, 5,000 and 10,000 r at 37.23 r per minute. The eggs were placed in the beam cross section 24 inches from the target in the X-ray tube, using 200 kilovolts and 20 milliamperes. Filters of 0.5 mm. of copper and 1.0 mm. of aluminum were used, in addition to 0.25 mm. of copper and 1.0 mm. of aluminum in the ionization chamber and approximately 0.25 copper and 1.0 aluminum inherent filtration of oil immersed therapy tube head at 200 kv. Temperature of the water varied from 6.1° to 6.7° C. during the exposures. Roentgens were measured in the

*This report is based on work performed under Contract No. W-28-094-eng-33 with the Manhattan District-Atomic Energy Commission.

TABLE I.
Mortalities in chinook salmon irradiated in eyed egg stages.

Dose in r	No. of specimens used	Number of deaths, in periods, in days after irradiation as indicated.														Total	Per Cent
		Days	23	30	37	44	51	58	65	72	79	86	93	100	107	114	
0	461		3	1	5	3	3	1	2	6	3	1	1	2	1		32	6.94
250	454		7		1		7	1		3	5	4	3	1	1	1	34	7.48
500	452		3	4	1	1	3			9	8	8	2		2		41	9.05
1000	440		8	3	6	22	29	13	5	5	9	62	42	21	5	1	231	52.5
2500	553		4	2	53	413	81										553	100
		Days	22	29	36	43	50											
5000	557		6	3		542	6										557	100
		Days	19	33		40	47											
10000	255		4			243	8										255	100

beam cross section in water by means of a Victoreen ionization chamber. The number of specimens for each exposure is listed in Table I.

During development the fish were kept in water temperatures averaging 9.9° C. and ranged from 6.1° to 15.0° C. These temperatures are well within the normal range for the race of chinook salmon used.

The chinook salmon eggs average about 8.7 mm. in diameter and have a specific gravity of about 1.011. Usually the embryo lies near the top of the egg so that very little water was interposed between the source of irradiation and the fish.

Samples for growth, development, and histological studies were taken of each experimental group, the first 24 hours after irradiation and at approximately weekly intervals thereafter. About nine fish per week were taken from each of the irradiated groups and a control group.

Part I. Gross Pathology

The fish in all experimental lots developed and hatched in an apparently normal fashion 12 to 16 days after irradiation. On about the 27th day after irradiation fish given doses of 10,000 and 5,000 r were observed to be less active than the other lots and on the 29th day fish irradiated with 2,500 r showed much the same symptoms. By the 33rd day these three lots suffered heavy mortalities after a somewhat characteristic syndrome. Most of these heavily irradiated

lots were unable to swim, the heart beat was slow and spasmodic, and many seemed unable to close or move the lower jaw normally in respiration. Vascular abnormalities were observable about four weeks after irradiation. The vitelline vein appeared abnormally distended and erythrocytes had invaded the subocular lymph sinuses (Fig. 1) which communicate by lateral pharyngeal vessels and lymphatico-venous valves with the anterior cardinal veins. According to McClure (1915) the communications are only temporary in trout during the embryonic stages, and, before the valves form, blood may sometimes flow freely into the lymphatics. The valves develop in trout embryos about 22 days old and the presence of red blood in the sinuses after this time indicates certain abnormalities which might be induced by rough handling, shock or treatment with chlorotone. This experiment indicates that chinook salmon embryos react similarly following exposure to X-rays.

About 30 days after exposure the vitelline vein appeared abnormal in fish irradiated with 1,000 r. A few fish in this lot appeared weak, had indications of subcutaneous hemorrhage along the lateral abdominal walls (Fig. 2) and contained blood in the subocular lymph sinuses. Otherwise, most of the fish irradiated with 1,000 r or less appeared normal.

By the 33rd day vascular damage was heavy in fish irradiated with 2,500 r or more. Subcutaneous extravasation was especially noticeable along the belly wall below the lateral line near the attachment of the yolk sac (Figs. 1 and 3) as well as along the lower jaw in practically all specimens in these groups. At about this same time white flocculent spots were noticeable in the yolks of these fish. Some specimens in the 1,000 r lot were similarly affected. This may be an indication of a denaturing effect similar to that occurring in albumen and proteins after irradiation (Clark, 1936).

Damage to the vascular system seems to be a common effect of Roentgen rays among animals. Rushton (1936) and Corbella (1930) observed similar abnormalities in trout. Butler (1936) reported severe damage to the young of amphibia, birds and mammals. Borak (1942) suggests that capillary damage might be explained as a direct action of radiation on the endothelial cells. However, the radiations seem to affect the valves separating the lymph and venous systems in salmon embryos, resulting in extensive hemorrhage.

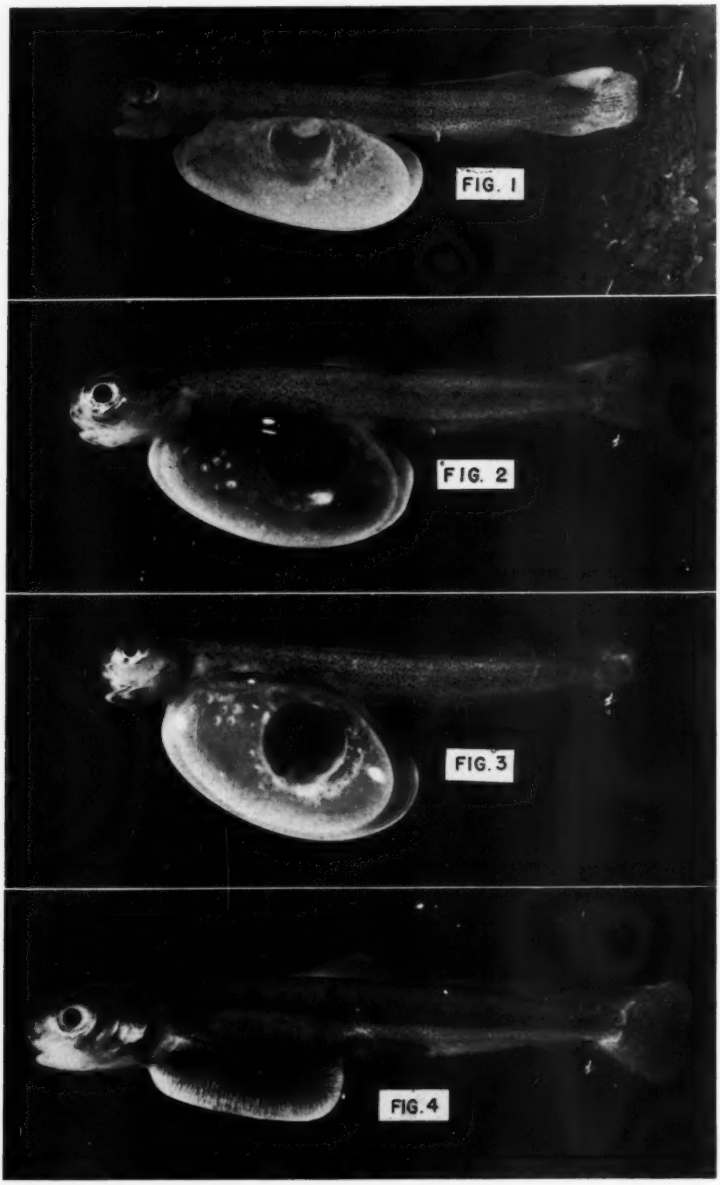


FIG. 1
FIG. 2
FIG. 3
FIG. 4

FIGURES 1, 2, and 3

are photographs of chinook salmon larvae 44 days after irradiation with 2,500, 1,000 and 2,500 r respectively and figure 4 is a photograph of a control fish of the same age. Note, in the irradiated specimens, the lack of development, the indications of edema, the presence of hemorrhage and other abnormalities as reported in the text. × 4.

Five weeks after exposure some of the fish in the lots irradiated with from 1,000 to 10,000 r seemed to lack red blood circulation in the vitelline vein or its branches (Figs. 1 and 3). Very little red blood seemed to be circulating, at least in the normal channels, in fish irradiated with 2,500 r or more. This was especially noticeable around the heart and gills. There were some instances of the accumulation of blood in the body cavity, drifting about in the yolk sac. In addition, an edematous condition was developing in these groups as indicated by an accumulation of serous fluid in the body cavity and around the eyes.

Only an occasional specimen in the fish irradiated with 1,000 r was observed to have edema. In fish exposed to 2,500, 5,000 and 10,000 r (Figs. 1, 2 and 3) the fluid accumulated between the inner and outer walls of the yolk sac and had a bluish tinge. The accumulation was frequently accompanied by fusion of fat droplets into a large globule. In many the sclerotic membrane of the eye was distended, a symptom which has been noted in the irradiated eyes of other animals (Warren, 1936).

Quantitative estimates of the amount of serous fluid in the body cavity were arrived at indirectly and graphed in Figure 5. The weight of the fluid was determined by comparing the weight of the larva, with yolk sac intact, with the weight of the larva and yolk after the serous fluid had been removed. In the three heavily irradiated lots there was a steady accumulation of serous fluid until death 40 to 50 days after exposure, and the rate of concentration was in proportion to the amount of radiation given.

The edematous condition may be related to the phenomenon produced by X-rays in proteins which lose their water-binding capacity after heavy doses (Clark, 1936). Loss of the water-binding capacity and, thus, the subsequent loss of weight of the yolk might explain the apparent absorption of yolk without consequent development of heavily irradiated larvae. The edema might also be due to damage to the pronephric tubules or to the pronephric glomerulus although no direct connection between these organs and the body cavity was seen in the larvae.

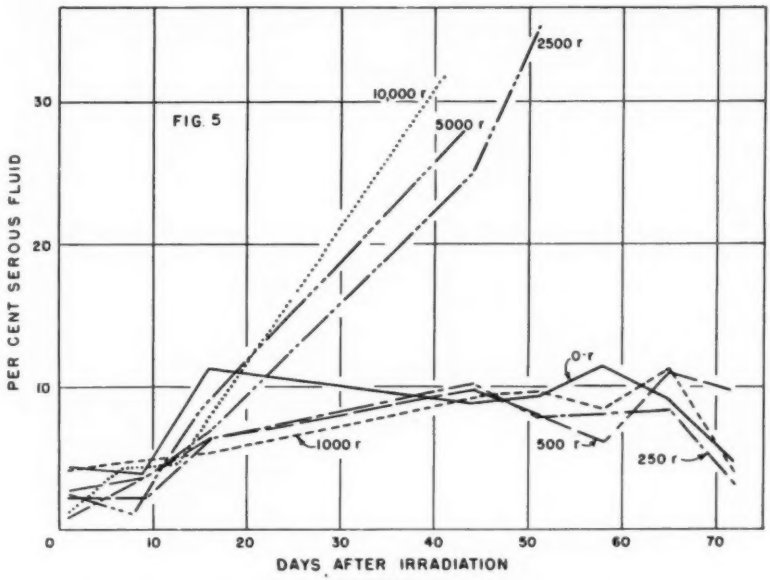


FIGURE 5.

Relationships of various amounts of roentgens and quantity of serous fluid present in the body cavity of chinook salmon to the days after irradiation. Data from Table V.

Mortalities

Most of the larvae in the lots irradiated with 2,500, 5,000 and 10,000 r died between the 30th and 50th days after exposure, with those irradiated with 5,000 and 10,000 r dying somewhat more rapidly (Table I, Fig. 6). Fish irradiated with 1,000 r were subject to accelerated mortalities during the same period. After the 37th day the cumulative mortalities in 1,000 r fish increased from 3.86% to 15.45% in 14 days. From the 53rd to the 79th day after exposure the mortality was comparatively normal, then increased rapidly to 51.13% by the 100th day after which the rate returned to normal until the 125th day when the experiment was terminated.

The possible causes of the mortalities during the period 30 to 50 days after irradiation will be discussed later. One of the factors that might have produced the second surge of mortalities was that the fish began active feeding about 70 days after irradiation. Those fish far-

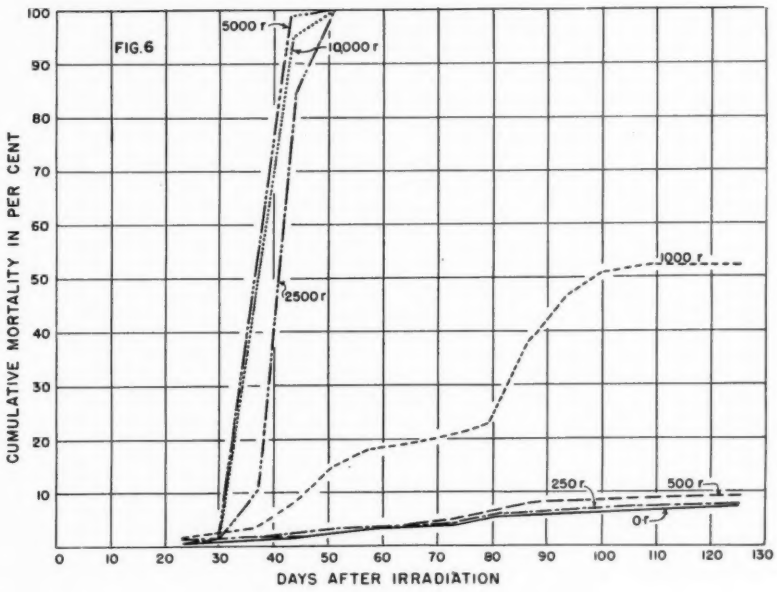


FIGURE 6.

Cumulative percentage distributions of mortalities during the 125 days following administration of various doses of X-rays to "eyed" embryos. Data from Table I.

thest along in development probably took in food within the next few days. Others, such as the 1,000 lot, whose development was obviously retarded and whose alimentary tract might be abnormal, very likely, did not start feeding until several days later, a time coincident with the beginning of the second period of increased death rate.

Chinook salmon irradiated with 500 r and lesser dosages did not differ significantly from the controls in the extent or rate of mortalities. Mortalities in the 500 r lot were 9.05% as compared with 7.48% in fish irradiated with 250 r and 6.94% in the controls of this group. There was an acceleration of mortalities of a minor nature in all these lots from the 71st to 86th day, remotely resembling that of 1,000 r fish, indicating a similar critical physiological period.

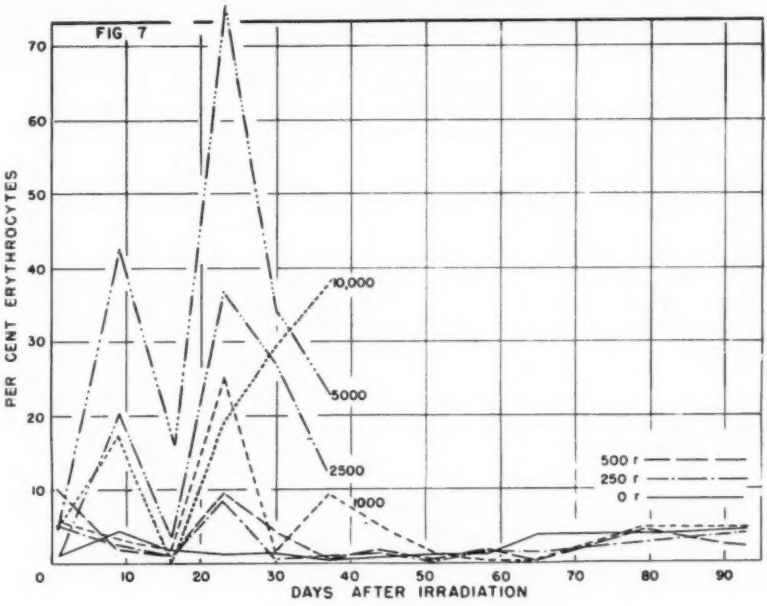


FIGURE 7.

Relationship of percentage of erythrocytes present in kidney hemopoietic tissue to days after administration of various doses of X-rays. Data from Table VI.

Growth

Analyses of lengths and weights by weekly periods were made on specimens preserved for histological and developmental studies. Standard lengths, the lengths from the tip of the snout to the base of the caudal fin rays, were measured to the nearest 0.1 mm. on all fish preserved after hatching, about 12 to 125 days after irradiation (Table II, Fig. 8). An analytical balance was used to determine wet weights (from 70% alcohol) of individual fish. The first sample was taken within 24 hours, and the rest at approximately weekly intervals. Egg shells were removed from the embryos and the embryos and larvae weighed, first with the yolk sac intact, then with the indurated yolk removed, and, finally, the yolk was weighed alone (Table II, Fig. 9).

Growth in length and weight were considerably retarded in the fish irradiated with 2,500 r or more although it seems evident that some

TABLE II.

STANDARD LENGTH (L) IN MM AND WEIGHTS (W) IN GRAMS (YOLK REMOVED) OF CHINOOK SALMON AFTER IRRADIATION IN EYED STAGES. TOTAL NUMBER OF SPECIMENS USED IN LENGTH CALCULATIONS WERE 614, AVERAGING 7.7 FISH PER SAMPLE. TOTAL NUMBER OF SPECIMENS USED IN WEIGHT CALCULATIONS WERE 325, AVERAGING 4.1 FISH PER SAMPLE.

Dose in r	Days*		1	9	16	23	30	37	44	51	58	65	72	79	86	93	100	107	114	125
0	L	$\bar{x}$			20.0	20.8	22.6	24.0	26.7	27.4	28.8	30.4	31.7	34.1	35.2	37.0	37.6	42.2	43.8	53.6
		s			.818	.346	.427	1.683	.565	1.117	1.029	.715	.637	1.845	1.056	1.723	1.876	3.844	2.237	2.644
	W	$\bar{x}$	.020	.024	.038		.070		.160	.210	.249	.313	.396	.538	.658	.770	.903	1.065	1.506	2.126
		s	.0062	.0033	.0041				.0048	.0512	.0565	.0393	.0522	.0842	.1013	.0608	.2481	.0655	.3047	.4380
250	L	$\bar{x}$			19.1	20.2	22.4	23.4	24.6	27.5	28.8	30.7	32.1	34.6	35.2	37.6	38.2	42.5	46.8	49.9
		s			.678	.391	.474	1.419	1.681	.718	.969	1.222	.945	1.392	1.553	1.316	1.905	2.296	3.045	3.197
	W	$\bar{x}$	.014	.029	.034		.057		.137	.205	.278	.288	.390	.574	.744	.783	.879	1.164	1.671	1.671
		s	.0024	.0029	.0058				.0281	.0382	.0549	.0422	.0340	.0890	.0372	.0526	.0234	.2821	.6772	.2189
500	L	$\bar{x}$			19.1	20.2	22.0	23.0	25.7	27.4	28.5	28.7	31.2	32.8	32.8	36.8	35.1	41.1	45.4	50.5
		s			.202	.300	.690	1.519	.608	.984	.616	1.500	.820	1.293	.912	2.630	1.446	2.868	3.038	5.039
		P			.01	.04	.08	.30	.01	1.00	.46	.01	.18	.10	.01	.85	.01	.46	.32	.20
	W	$\bar{x}$	.012	.026		.036	.062		.145	.206	.270	.262	.331	.540	.558	.921	.588	1.068	1.683	1.793
		s	.0016	.0034	.0053				.0080	.0310	.0516	.0664	.0342	.0329	.1017	.1574	.1023	.1386	.4038	.3936
		P	.01	.38	.52				.16	.89	.58	.22	.05	.97	.22	.20	.12	.97	.41	.18
1000	L	$\bar{x}$			18.8	20.0	22.1	23.2	25.1	26.6	27.7	29.3	29.7	29.9	31.6	33.4	34.2	37.6	35.9	43.7
		s			.369	.332	.219	1.337	.752	.874	.610	1.020	.766	.774	.704	1.332	1.126	4.554	.800	3.988
		P			.01	.02	.03	.38	.01	.11	.01	.02	.01	.01	.01	.01	.01	.02	.01	.01
	W	$\bar{x}$	.015	.027	.036		.065		.146	.184	.226	.260	.278	.420	.451	.549	.572	.637	.809	1.164
		s	.0034	.0027	.0039				.0268	.0335	.0324	.0357	.0090	.0405	.0564	.1326	.0897	.1266	.1562	.3413
		P	.16	.17	.46				.54	.37	.47	.08	.01	.06	.01	.06	.10	.01	.01	.01
2500	L	$\bar{x}$			19.0	19.8	21.4	20.9	23.6	23.8										
		s			.214	.342	.520	.527	.662	.224										
	W	$\bar{x}$	.014	.029	.036		.051		.108	.122										
		s	.0034	.0036	.0060				.0091	.0571										
	Days*		1	8	15	22	29	36	43											
5000	L	$\bar{x}$			18.7	19.7	20.9	20.4	22.5											
		s			.572	.173	.550	.736	.440											
	W	$\bar{x}$	.012	.026	.032		.056		.074											
		s	.0012	.0017	.0038				.0078											
	Days*		1	5	12	19	26	33	40											
10000	L	$\bar{x}$			18.4	19.4	20.2	20.0	22.3											
		s			.454	.594	.634	.307	.567											
	W	$\bar{x}$	.016	.021	.033		.051		.075											
		s	.0064	.0028	.0075				.0064											

*Indicates the number of days after irradiation.

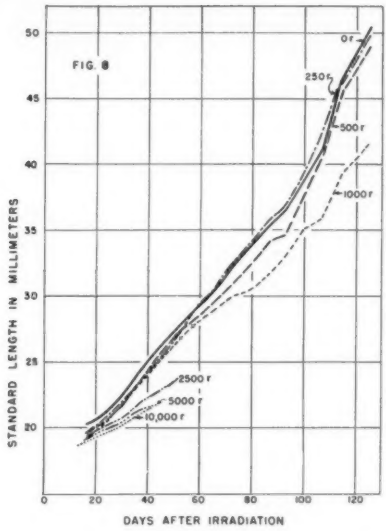


FIGURE 8.
Relationship of average lengths of chinook salmon larvae to days after administration of various doses of X-rays. Data from Table II, smoothed by a moving average of three.

growth took place in spite of the heavy dosages. This retardation compares roughly to the amount of irradiation given. The length measurements in larvae exposed to 1,000 r were significantly less than the controls at least by the 58th day after exposure as measured by the "t" test and probability values, "P" (Simpson and Roe, 1939). P values refer to the probability of the lot being a sample from a population such as the control. Differences in the weights of these two lots of fish as determined by the probability value P (Table II), are slight and doubtful through the 65th day after irradiation. On the 125th day the salmon irradiated with 1,000 r averaged about 45% lighter in weight and 18.5% shorter in length than the controls.

The means of the lengths reveal a temporary delay in growth of larvae irradiated with 500 r. Probability values varied from 0.01 to 1.00, averaging about 0.10 and Figure 8 reveals a slight lag in growth beginning about the 60th day after irradiation and ending about the 100th day.

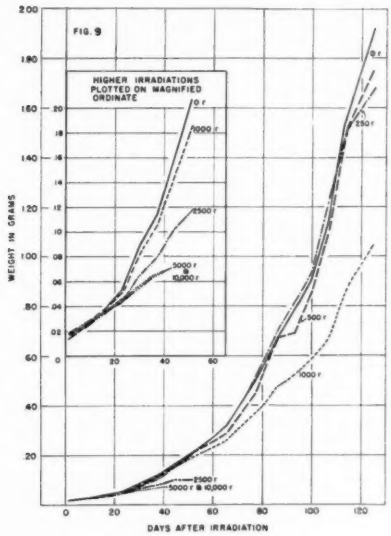


FIGURE 9.
Relationship of average weights of chinook salmon embryos and larvae to days after irradiation with various amounts of X-rays. Data from Table II, smoothed by a moving average of three.

No significant differences were detected in the weights of specimens irradiated with 500 r and no significant differences were detected in the lengths and weights of specimens irradiated with 250 r when compared with nonirradiated fish.

A number of live fish were also weighed and a summary of the results of the live-fish weighings is given in Table III which should be compared with Table II and Figure 9.

TABLE III.
AVERAGE WEIGHTS IN GRAMS OF CHINOOK SALMON FOLLOWING EXPOSURES TO X-RAYS. EACH WEIGHT REPRESENTS AN AVERAGE OF 200 FISH.

Days after Irradiation	Control 0 r	250 r	500 r	1,000 r
77	0.685	0.710	0.705	0.565
91	0.915	0.990	0.965	0.675
105	1.420	1.400	1.385	0.925
119	2.085	2.100	2.105	1.365

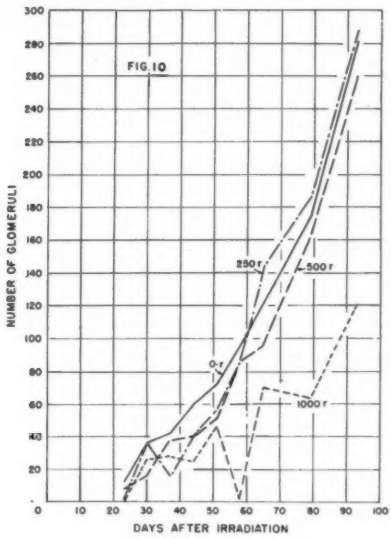


FIGURE 10.
Relationship of the number of glomeruli in the kidneys of chinook salmon larvae to days after irradiation with various doses of X-rays. Data from Table VII.

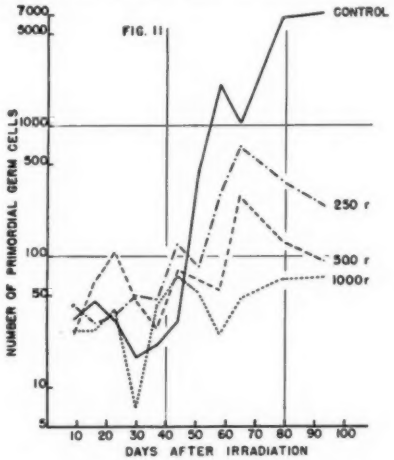


FIGURE 11.
Relation of the number of primordial germ cells to the days after irradiation for control and irradiated chinook salmon larvae. Data from Table IX.

Differences between Tables II and III in the average weights of samples taken at the same time and from the same lot can be attributed to the differences in methods of weighing. In the process of live-fish weighing a small amount of water is unavoidably weighed along with the fish and the resultant weight is slightly higher than the true weight of the fish. Further, wet weights from 70% alcohol will probably be smaller than the true weight of the fish when we consider that the specimens were saturated with alcohol which has a slightly lower specific gravity than water, and also that evaporation taking place during the weighing process could account for the lower average weights in Table II.

Development

The comparative slowness or lack of pigment development in irradiated as compared with control fish was noticeable about 24 days after exposures to 2,500, 5,000 and 10,000 r. Although the chromatophores were somewhat more expanded in irradiated fish there were fewer chromatophores present than in the controls. This lack of pigment was one of the first evidences that development was being retarded in X-rayed fish. Other evidences soon followed, as in the development of the fins and eyes and the comparative sizes of the yolk sac. None of the fins developed appreciably during the six-week period following irradiation in fish exposed to 2,500, 5,000 and 10,000 r. At this time the dorsal, caudal and anal fins of these fish had only short, feebly developed fin rays and most of the fins were more or less joined together by the embryonic fin fold. In nonirradiated fish the fin rays were completely developed to the edge of the fin and the fin fold was absorbed except between the ventral fins and anus, and on the caudal peduncle (Figs. 1, 2, 3 and 4). Development of ventral and pectoral fins was similarly retarded.

Inhibition of fin development was not so noticeable in fish irradiated with 1,000 r, but some showed incompletely developed anal rays and a somewhat more juvenile appearance in the caudal and dorsal fins than nonirradiated fish 44 days after exposure. The fins of fish irradiated with 500 and 250 r appeared normal.

Retardation of the development of the pigment was evident 40 days after exposure in some of the fish irradiated with 1,000 r while other specimens of this group appeared similar to nonirradiated fish. The

TABLE IV.

DIAMETER OF THE EYE (IN MM) OF CHINOOK SALMON EMBRYOS AND LARVAE AFTER IRRADIATION IN EYED STAGES. A TOTAL OF 299 SPECIMENS WAS USED AVERAGING 3.7 PER SAMPLE.

Dose in r	Days*	1	9	16	30	44	51	58	65	72	79	86	93	100	107	114	125
0	$\bar{x}$	1.40	1.44	1.74	1.90	2.30	2.47	2.70	2.88	3.07	3.08	3.18	3.47	3.27	3.62	3.75	4.12
	s	.000	.089	.055		.283	.153	.141	.036	.058	.117	.171	.058	.058	.171	.164	.117
250	$\bar{x}$	1.25	1.58	1.64	1.70	2.10	2.52	2.80	2.95	3.12	3.20	3.12	3.27	3.47	3.77	3.84	3.93
	s	.217	.148	.055		.141	.104	.000	.071	.126	.100	.050	.579	.058	.404	.288	.111
500	$\bar{x}$	1.27	1.50	1.64	1.70	2.15	2.10	2.70	2.93	3.02	3.03	3.05	3.47	3.40	3.52	3.75	3.97
	s	.138	.100	.055		.071		.283	.153	.126	.058	.100	.153	.361	.050	.176	.095
	P	.25	.35	.02		.53	.10	1.00	.69	.56	.52	.23	1.00	.57	.91	1.00	.03
1000	$\bar{x}$	1.20	1.48	1.65	1.90	2.18	2.33	2.53	2.70	2.83	2.83	2.95	3.03	3.07	3.28	3.35	3.80
	s	.173	.084	.094		.425	.226	.295	.173	.153	.153	.058	.116	.058	.130	.084	.173
	P	.17	.49	.10		.75	.42	.52	.27	.07	.03	.04	.01	.01	.01	.01	.01
2500	$\bar{x}$	1.20	1.44	1.50	1.50	1.50	1.30										
	s	.122	.089	.071		.283											
	Days*	1	8	15	29	43											
5000	$\bar{x}$	1.40	1.37	1.35	1.30	1.37											
	s	.058	.084	.100		.153											
	Days*	1	5	12	26	40											
10000	$\bar{x}$	1.35	1.55	1.29	1.50	1.55											
	s	.071	.071	.258		.071											

*Indicates the number of days after irradiation.

$\bar{x}$ refers to the arithmetic mean.

FIG. 12

FIG. 13

FIG. 14

FIGURES 12, 13 and 14

are photomicrographs of 10-micron sagittal sections through the heads of larvae 37 days after irradiation with 0 r, 1,000 r and 500 r respectively. Note the comparative development of the gills and the accumulation of red blood cells in the subocular lymph sinus in figure 13. × 18.

development of silvery pigment was definitely inhibited as indicated in these figures. From the 58th to the 72nd day after irradiation the fish in this lot continued to lag behind in the development of coloration. Some slight differences in pigmentation were noticed in 500 r fish about 37 days after irradiation, apparently only of a very temporary nature. Sparse but expanded chromatophores were evident at this time. There was apparently no development of the coloration in fish of the three heavily exposed groups during the time of their survival.

The effects of X-rays on the melanophores of salmon parallel only slightly the effects seen in goldfish by Smith (1932), Ellinger (1939), Ellinger and Davison (1942) and Davison and Ellinger (1942). There is only a slight expansion of the chromatophores occurring in the chinook salmon after irradiation.

The development of the eye, as determined by its diameter measured parallel to the body axis, progressed similarly to that of the length and weight in irradiated fish. Very little, if any, increase in diameter took place in the three heavily irradiated lots, 2,500, 5,000 and 10,000 r (Table IV). In some cases the opaque condition of the lens indicated that the specimens may have been blinded by severe dosages. In fish irradiated with 1,000 r the differences in regard to the diameter of the eye from those of the controls were found to be significant after the 79th day following exposure.

Retardation of development or destruction of gill lamellae was demonstrated in fish given dosages of 1,000 r or more (Compare Figs. 12 and 13), while an occasional fish exhibited retardation of gill filament growth after irradiations of 500 r (Fig. 14). Lethal doses of 2,500 r or more almost completely destroyed or inhibited gill development.

Irradiations of 1,000 r distorted the gill lamellae sufficiently to render this group distinguishable from the others by examination of the gills. The lamellae were seen to have an uneven or deformed character in contrast with the regular nature of control gills.

Irrespective of the probable effects of X-rays directly on the yolk and irrespective of the amount of growth or development taking place in the larvae, the yolk material appeared to be absorbed at about the same rate in all the experimental lots (Table V).

TABLE V.

WEIGHTS IN GRAMS OF CHINOOK SALMON EMBRYOS AND LARVAE WITH YOLKS (W) AND WEIGHTS OF THE YOLK ALONE (Y) ALL TAKEN AFTER IRRADIATION IN EYED STAGES. A TOTAL OF 200 FISH WERE USED AVERAGING 4.4 FISH PER SAMPLE.

Dose in r	Days*	W 1	Y 1	W 9	Y 9	W 16	Y 16	W 44	Y 44	W 51	Y 51	W 58	Y 58	W 65	Y 65	W 72	Y 72
0	$\bar{x}$	.272	.244	.217	.185	.284	.214	.353	.162	.382	.136	.395	.100	.408	.058	.439	.022
	s	.0149	.0184	.0058	.0265	.0696	.0175	.0199	.0202	.0794	.0245	.0538	.0182	.0298	.0259	.039	.0094
250	$\bar{x}$	.248	.229	.253	.218	.283	.231	.348	.175	.378	.143	.406	.095	.374	.054	.438	.035
	s	.0378	.0394	.0473	.0437	.0095	.0137	.0314	.0278	.0451	.0219	.0727	.0224	.0362	.0164	.0622	.0268
500	$\bar{x}$	.258	.239	.252	.217	.251	.199	.340	.162	.372	.136	.381	.088	.367	.066	.399	.029
	s	.0160	.0160	.0196	.0209	.0445	.0355	.0426	.0353	.0505	.0200	.0583	.0149	.0467	.0166	.0276	.0132
1000	$\bar{x}$	.242	.217	.240	.201	.276	.225	.366	.186	.350	.132	.366	.109	.372	.070	.345	.053
	s	.0269	.0400	.0128	.0524	.0339	.0339	.0419	.0409	.0430	.0174	.0448	.0189	.0332	.0196	.0423	.0155
2500	$\bar{x}$	.248	.232	.240	.202	.289	.233	.368	.168	.507	.207						
	s	.0391	.0384	.0204	.0222	.0317	.0212	.0734	.0291								
	Days*	1	1	8	8	15	15	43	43								
5000	$\bar{x}$	.248	.230	.237	.208	.261	.207	.339	.170								
	s	.0285	.0308	.0214	.0217	.0363	.0252	.0546	.0132								
	Days*	1	1	5	5	12	12	40	40								
10000	$\bar{x}$	.265	.246	.225	.194	.268	.222	.358	.170								
	s	.0481	.0559	.0156	.0134	.0011	.0042	.0388	.0237								

*Indicates the number of days after irradiation.

$\bar{x}$ refers to the arithmetic mean.

FIG. 15

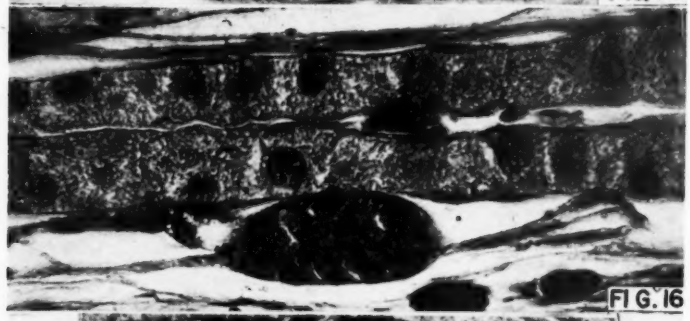


FIG. 16

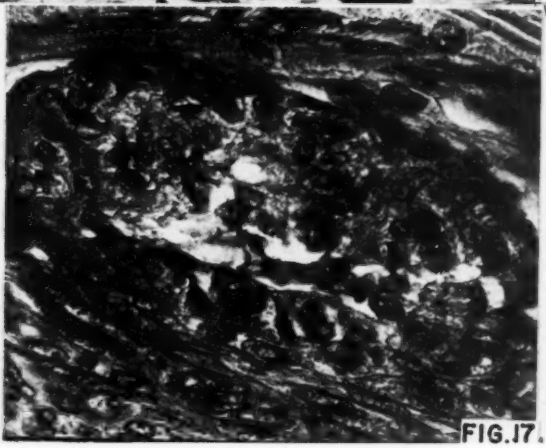


FIG. 17.

FIGURES 15 and 16

are photomicrographs of a five-micron sagittal section through the interrenal organ of a control (Fig. 15) and an irradiated (10,000 r, Fig. 16) larva 40 days after treatment. × 680.

FIGURE 17.

Photomicrograph of a five-micron sagittal section of the spleen of a chinook salmon larva 44 days after treatment with 2,500 r. × 680.

Part II. Histopathology

Methods

Histological studies were made on serial sagittal sections of a total of 84 embryos and larvae killed at weekly intervals during the course of the experiment. As much of the yolk as possible was removed in order to facilitate sectioning, and since no vital organs, except, perhaps, the caudal lymphatic heart, existed in the posterior region of the body, only the anterior part of the body as far back as the anus was sectioned for study. All the sections were cut five microns thick and stained with hematoxylin and triosin in as uniform a procedure as possible.

Counting of cells was done by projecting the section to be counted on a ground glass screen fitted with a grid calibrated to correspond to a 50 by 50 micron area of tissue under the microscope. Many of the counts were checked by direct examination under the microscope. This method was necessary also to verify doubtful stages of mitosis and to distinguish between certain types of degenerate and normal cells. Whole organs, or certain definitely located areas in the organs, were counted. For example, in the counts of the hemopoietic cells of the kidney the mid-line of the organ was located by the presence of the pronephric glomerulus and the coeliaco-mesenteric artery. Then, starting at the anterior end of the kidney in this sagittal section, five successive 50 by 50 micron areas were counted each over a different part of the kidney so that the fifth area counted was just posterior to the coeliaco-mesenteric artery. In addition, several counts, usually five in all, were made on every other section cut on each side of the mid-line of the kidney and in the region just posterior to the coeliaco-mesenteric artery. Thus, for any one kidney at least 10 areas, 50 microns square, were counted giving a total volume of about 125,000 cubic microns.

Hemopoietic Tissue

After a cursory examination of the organs of all the specimens prepared during the course of the experiment it became evident that the hemopoietic tissue located in the kidney was injured as much, if not more than, any other tissue by exposure to X-rays. Furthermore, the sensitivity of the hemopoietic tissue to Roentgen rays is well-known in the case of other animals (Warren, 1936; Dunlap, 1942; etc.).

In teleosts in general the intertubular tissue of the kidney is the predominant hemopoietic organ, with the spleen of secondary importance. There are apparently no lymph nodes to supplement the activities of these two organs although under certain conditions the intestinal mucosa may become hemopoietic in some fishes (Jordan and Speidel, 1924).

The kidney in the chinook salmon is an elongate organ lying beneath the backbone and extending from the 4th to the 41st vertebra, about the length of the body cavity. Internally, in embryos and larvae, a large pronephric glomerulus lies in the anterior part of the kidney (Fig. 21), tubules are present here in early development but later disappear. The rest of the kidney is largely hemopoietic except for the paired pronephric tubules, the post-cardinal veins and a few small spherical inter-renal bodies. Soon after hatching Malpighian corpuscles and associated tubules begin to develop in the posterior part of the kidney, usually in the area beneath vertebrae 24 to 33. As the larvae develop, more glomeruli appear anterior and posterior to this area and increase in number to extend throughout the posterior two-thirds of the kidney. During this time the pronephric glomerulus and tubule begin to degenerate, so that in the fingerling stages the pronephric kidney is mainly hemopoietic in function.

Figure 18 is a graph of the cell counts (Table VI) smoothed by a moving average of three. The figure reflects almost the same situation that existed in the previous studies of mortalities, lengths, etc., relative to the effects of similar doses. The fish irradiated with 2,500 r or more showed a reduction in the number of hemopoietic cells from about 43 to about 15 per 12,500 cubic microns of tissue (average of the 10 or more 50 by 50 micron areas counted in each fish). The curve of the cell counts of fish irradiated with 1,000 r again takes up an intermediate position, the counts averaging as low as 31.5 per 12,500 cubic microns 23 days after irradiation, after which recovery apparently took place.

Exposures to 500 r X-rays reduced the numbers of hemopoietic cells so that the count was below and more or less significantly different from the controls (as measured by P) for the first 50 days following irradiation. Recovery in this lot was approximately 12 days earlier than in the 1,000 r lot and about 10 days later than the lot irradiated with 250 r. The 500 and 250 r lots both show a slight

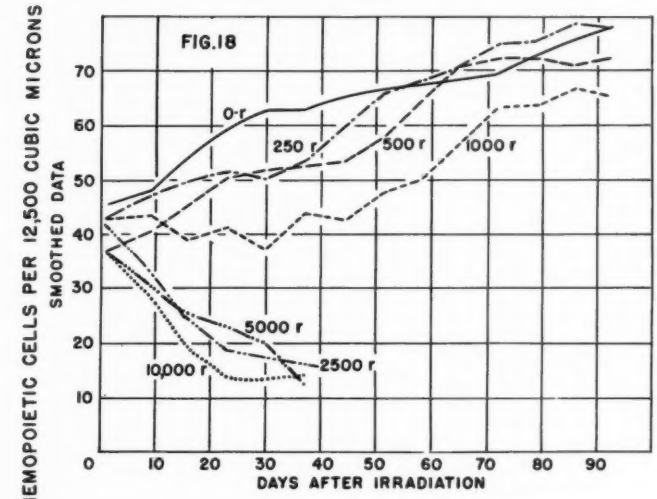


FIG.18
HEMOPOIETIC CELLS PER 12,500 CUBIC MICRONS
SMOOTHED DATA
0 r
250 r
500 r
1000 r
5000 r
2500 r
10,000 r
DAYS AFTER IRRADIATION

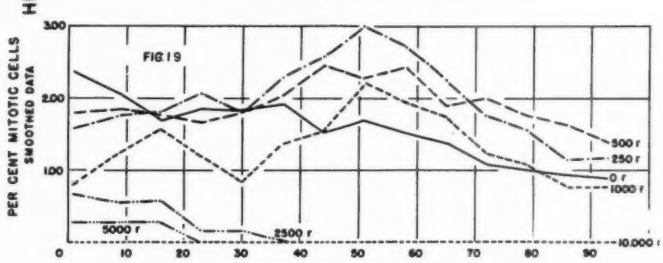


FIG.19
PER CENT MITOTIC CELLS
SMOOTHED DATA
500 r
250 r
0 r
1000 r
5000 r
2500 r
10,000 r

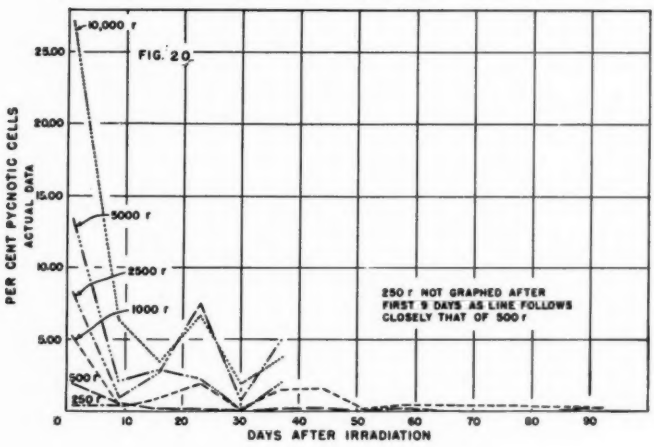


FIG. 20
PER CENT PYCNOTIC CELLS
ACTUAL DATA
10,000 r
5000 r
2500 r
1000 r
500 r
250 r
250 r NOT GRAPHED AFTER FIRST 9 DAYS AS LINE FOLLOWS CLOSELY THAT OF 500 r
DAYS AFTER IRRADIATION

FIGURE 18.

Relation of the number of hemopoietic cells in the kidney tissue of chinook salmon embryos and larvae to the days after irradiation with X-rays. Data from Table VI.

FIGURES 19 and 20.

Relation of the percentage of mitotic cells (Fig. 19) and pycnotic cells (Fig. 20) in the hemopoietic tissue of the kidney to the days after irradiation with X-rays. Data from Table VI.

TABLE VI.

AVERAGES OF CELL COUNTS MADE IN 12,500 CUBIC MICRONS OF HEMOPOIETIC TISSUE OF KIDNEY WITH PERCENTAGES OF ERYTHROCYTES, MITOTIC CELLS AND DEGENERATE CELLS IN THESE COUNTS. MEANS AND PERCENTAGES REPRESENT AVERAGES OF 10 COUNTS PER SPECIMEN. SIXTY-SIX SPECIMENS ARE REPRESENTED.

Dose in r	Days*	1	9	16	23	30	37	44	51	58	65	79	93
0	$\bar{x}$	44.3	45.9	53.4	65.4	59.5	63.6	65.9	66.7	67.1	68.0	70.4	80.2
	s	2.53	4.04	4.50	12.1	3.66	7.26	8.86	4.83	3.82	6.88	7.71	2.78
	Erythrocytes	1.12	4.36	1.87	1.07	1.34	0.31	0.76	1.05	1.04	3.97	3.83	4.61
	Mitosis	2.46	2.18	1.50	1.38	2.69	1.42	1.67	1.50	1.89	1.18	0.99	0.87
	Degenerate	0	0	0	0	0	0	0	0	0	0	0	0
250	$\bar{x}$	43.1	42.9	55.0	52.2	47.7	50.9	62.1	66.8	68.0	69.0	80.1	76.4
	s	6.59	5.20	5.40	10.3	7.59	7.37	7.40	4.64	6.00	6.68	7.71	4.65
	P	.458	.148	.482	.021	.002	.003	.314	.644	.666	.727	.012	.042
	Erythrocytes	5.10	2.33	0.91	8.43	0.63	0.98	1.45	0.15	1.62	1.45	2.87	3.93
	Mitosis	1.62	1.49	2.18	1.72	2.31	1.38	3.22	3.14	2.65	2.38	1.12	1.18
	Degenerate	0.46	0.42	0.18	0.38	0.21	0.39	0.16	0.15	0	0	0	0.13
500	$\bar{x}$	36.5	37.5	46.8	53.2	50.8	51.6	54.7	53.3	64.4	74.8	69.7	72.1
	s	5.30	4.53	4.69	10.5	6.78	5.08	5.16	4.14	7.15	5.77	3.37	5.67
	P	.01	.01	.01	.030	.01	.01	.01	.01	.269	.031	.796	.01
	Erythrocytes	9.97	1.87	1.07	9.56	4.33	0.58	1.64	0.38	1.71	0.27	4.59	2.64
	Mitosis	1.37	2.67	1.50	1.13	2.36	1.94	1.83	3.00	1.71	2.00	2.01	1.25
	Degenerate	1.97	0.53	0.21	0.19	0	0.19	0.18	0	0.16	0	0	0.14
1000	$\bar{x}$	44.6	41.3	45.0	31.5	47.5	33.2	51.0	43.5	48.7	57.8	68.7	63.9
	s	5.97	6.57	8.06	6.93	6.19	6.07	6.02	5.38	6.38	9.51	5.21	4.01
	P	.301	.078	.010	.01	.01	.01	.01	.01	.01	.014	.571	.01
	Erythrocytes	5.83	3.39	1.78	25.08	1.47	9.64	5.10	1.61	0.41	0.17	4.66	4.85
	Mitosis	0	2.42	1.33	0.95	1.26	0.30	2.55	1.84	2.26	1.73	0.73	0.78
	Degenerate	5.22	0.24	0.89	1.90	0.21	1.51	1.57	0.23	0.41	0.35	0.29	0.16
2500	$\bar{x}$	46.3	31.5	22.0	21.3	13.5	18.0						
	s	7.09	4.67	5.56	6.90	4.88	4.24						
	Erythrocytes	0.78	20.32	3.18	36.62	26.67	11.67						
	Mitosis	0.39	1.27	0	0.47	0	0						
	Degenerate	8.25	0.95	2.73	7.51	0.74	5.00						
	Days*	1	8	15	22	29	36						
5000	$\bar{x}$	43.6	23.6	24.3	27.0	18.3	14.7						
	s	6.54	4.06	7.70	3.97	2.06	3.71						
	Erythrocytes	4.59	42.37	16.05	75.56	34.4	23.13						
	Mitosis	0	0.85	0	0	0	0						
	Degenerate	13.53	2.12	2.88	2.22	0	2.04						
	Days*	1	5	12	19	26	33						
10000	$\bar{x}$	41.9	27.1	17.5	13.6	10.5	16.0						
	s	5.36	6.28	4.87	6.95	4.17	6.16						
	Erythrocytes	5.25	17.45	0	19.12	29.52	38.12						
	Mitosis	0	0	0	0	0	0						
	Degenerate	27.21	6.38	3.43	6.62	1.90	3.75						

stimulation above normal after recovery similar to the stimulation noted in the bone marrow of rabbits (Mardersteig, 1938).

A graph of the percentages of erythrocytes of the total number of cells counted in the hemopoietic tissue (Fig. 7) indicates a considerable, although similar, variation in most of the lots exposed to X-rays. However, the fish exposed to 10,000 r digressed from the trend after the 20th day and the lot exposed to 1,000 r digressed between the 30th and 40th day after irradiation. In most of the lots the amount of roentgens had a similarly variable but directly proportional influence on the percentages of erythrocytes. It must be borne in mind, however, that the numbers of hemopoietic cells present are being reduced, and, although there is an actual increase in erythrocytes per volume of tissue in irradiated fish, the increase is accentuated by treating the data as percentages. Furthermore, there are at least two ways in which erythrocytes might find their way into kidney hemopoietic tissue. One is that the erythropoietic cells probably present are continually giving rise to red blood cells; another source would be through the infiltration of red blood through the capillaries associated with the renal portal system. In any case the presence of an excess of erythrocytes might be due partly to the breakdown of vessels in the kidney as well as to the continued maturing of erythrocytes whose precursors were presumably uninjured by exposure to Roentgen rays. Several observers have noted that irradiation produces a wave of red cell regeneration followed by prolonged increase in red cell count (Dunlap, 1942).

Definite reduction in the percentage of mitotic cells in the hemopoietic tissue in proportion to the amount of dose was evident in the lots exposed to 1,000 r or more. In general Figure 19 illustrates the well-known hypothesis that Roentgen rays inhibit cell division, and, in heavy dosages, arrest it permanently. Fish exposed to 1,000 r or less evidently recovered in the 40 days following exposure. Thereafter an acceleration of mitotic rate took place in these lots, roughly in proportion to the amount of dosage, which reached a peak about 50 days after irradiation.

It is of interest to note the general downward trend of the rate of mitosis in the control fish as development proceeded. This might presume a reduction of mitotic activity with age and development in the kidney hemopoietic tissue.

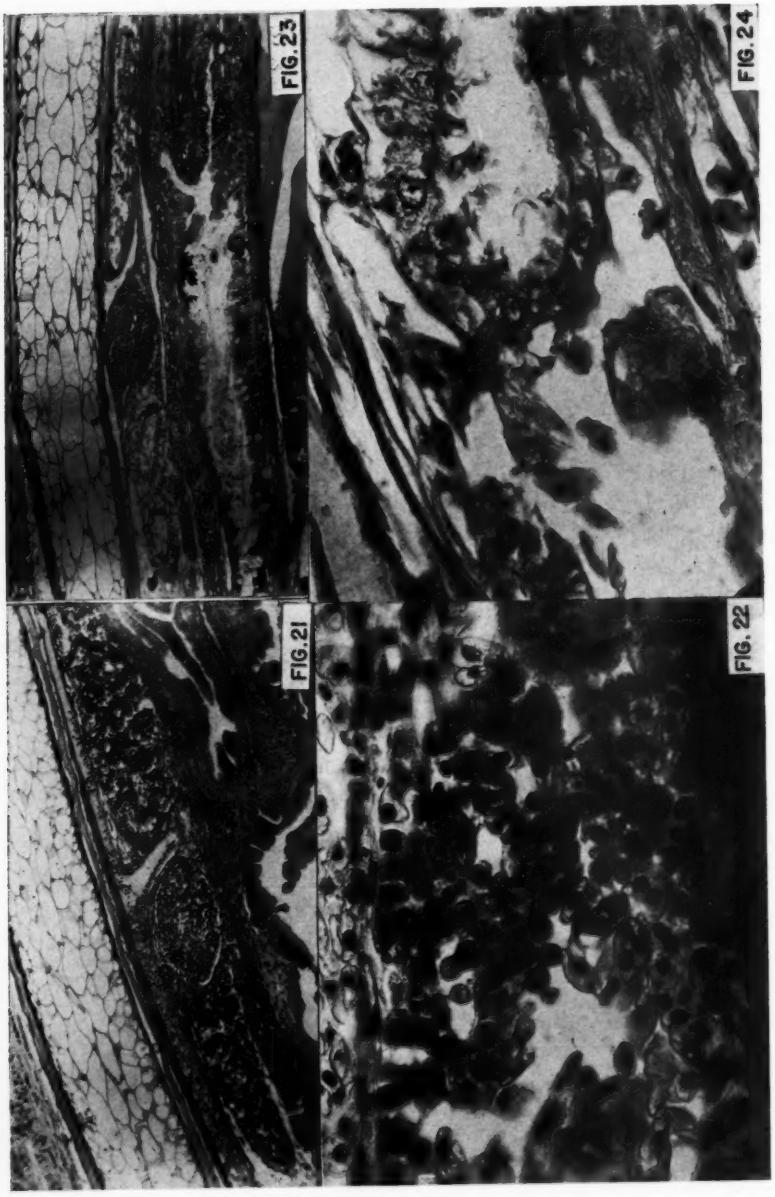


FIG. 23
FIG. 24
FIG. 21
FIG. 22

FIGURE 21.

Photomicrograph of a five-micron medial sagittal section of a chinook larva control. The organs are, from top to bottom, spinal cord, notochord, dorsal aorta, kidney (with ovoid pronephric glomerulus and ventrally slanting coeliaco-mesenteric artery) and alimentary tract. × 60.

FIGURE 22.

Photomicrograph of a medial sagittal section through the pronephros of a salmon embryo 24 hours after exposure to 5,000 r. Note the pycnotic nuclei. × 560.

FIGURE 23.

Photomicrograph of a medial sagittal section of a chinook larva 43 days after exposure to 5,000 r. Compare with figure 21. × 60.

FIGURE 24.

Photomicrograph of a medial sagittal section through the pronephros of a chinook larva 44 days after exposure to 2,500 r. × 560.

Practically the only degenerative cell types which could be distinguished readily and counted with confidence were cells undergoing pycnosis. Some nuclei underwent fragmentation and were distinguishable at some stages. These fragmented nuclei and other recognizable degenerative types were counted with the pycnotic cells and are included under this general term. Because of the difficulty in recognizing all degenerative types the counts are presumably lower than actually was the case.

The percentage of cells undergoing pycnosis was greatest in irradiated fish sacrificed 24 hours after exposure (Figs. 20 and 22), with lesser but variable pycnotic activity thereafter, all in proportion to the amounts of radiation given. No definite pycnotic nuclei were seen in nonirradiated specimens. The lethal effects continued at a moderate rate in fish irradiated with 2,500, 5,000 and 10,000 r with no apparent recovery leaving only some reticular stroma at death (Figs. 23 and 24). Cells in the 1,000 r lot were undergoing pycnosis at a low rate for 93 days, and fish irradiated with 500 and 250 r had an occasional pycnotic cell present through the 58th day or longer (Table VI).

Glomeruli

Counts of glomeruli (Table VII) were made by making tracings of the position of each glomerulus in the sagittal sections, in relation to the vertebrae immediately above. This method minimized the danger of counting a renal corpuscle more than once, served to delineate their distribution, and revealed the extension of glomeruli throughout all parts of the kidney as development proceeded.

The pronephric glomerulus was present and well developed in the head kidney at the time of irradiation, and subsequent observation did not reveal direct damage by X-rays. Roentgen sensitivity did not appear very great in this organ and much of the degeneration ap-

TABLE VII.
COUNTS OF THE GLOMERULI IN THE KIDNEY OF CHINOOK SALMON LARVAE AFTER IRRADIATION IN EYED EGG STAGES. COUNTS MADE ON 36 FISH.

Dose in r	Days	23	30	37	44	51	58	65	79	93	Average
0		12	36	42	60	66	98	122	174	282	99.1
250		2	38	16	41	48	86	144	186	288	94.3
500		8	16	38	40	51	86	96	162	260	84.1
1000		0	26	28	25	46	2	70	64	120	42.3

pearing at 37 days in fish given lethal doses can be attributed to cachexia.

The Malpighian corpuscles of the mid-kidney began to develop in non-irradiated salmon in the area below the 24th to the 33rd vertebra about one week after hatching or 23 days after irradiation. The number of glomeruli increased fairly rapidly in normal fish so that by the 93rd day there were about 282 in the mid- and hind-kidney. At this time none had developed anterior to the 12th or posterior to the 40th vertebra. Most of the glomeruli were concentrated in an area anterior to the ureter which emerges ventrally from the kidney usually below the 37th vertebra.

No glomeruli developed after irradiation with 2,500 r or more. Figure 10 illustrates the development of glomeruli in fish irradiated up to 1,000 r and indicates that 1,000 r definitely inhibited the development of glomeruli to the extent that by the 93rd day these specimens had roughly less than half the number of the control fish of the same age. Development was only slightly inhibited in salmon irradiated with 500 r while 250 r fish showed slight hypoplasia to the 60th day. Hyperplasia of glomerular development, corresponding roughly to the stimulation seen in the hemopoietic cells and growth in weight and length was found in fish irradiated with 250 r after the 60th day.

No great damage to the pronephric tubules or other kidney ducts was noticed until complete necrosis just after death in fish irradiated with 2,500 r or over (Figs. 23 and 24). Some nuclear pycnosis was seen 24 hours after exposure and some degeneration was evident 16 days later in specimens exposed to 10,000 r and 23 days later in specimens exposed to 5,000 and 2,500 r. Mesonephric tubules failed to develop in these specimens although anlagen were present at the time of irradiation. The anlagen persisted for some time then showed degeneration in fish treated with 10,000 and 5,000 r about five weeks after exposure and in six weeks in fish irradiated with 2,500 r. Some damage was noted in mesonephric ducts of 1,000 r fish about six weeks after exposure. In general, the damage seems to have been only indirectly caused by X-rays in these lots.

Interrenal Bodies

The interrenal bodies, or corpuscles of Stannius, develop as buds from the mesonephric duct in the mid-kidney area, usually below the

18th to 22nd vertebra in chinook salmon. These organs are present in the eyed embryos as one to three pairs of segmentally arranged ovoid bodies. The nuclei of the cells are relatively large and the cells are arranged in strands or in stratified semi-concentric layers (Fig. 15). Usually there is a variation in the diameter of the bodies in a 40 mm. salmon from about 50 to 160 microns. As the fish develops the number of pairs of interrenal organs increases to as much as six, although the average appears to be about four in chinook salmon of standard lengths of about 50 mm.

The interrenal bodies, like the adrenal glands of higher animals, seem to be fairly resistant to X-rays (Warren, 1936). Some damage due to irradiation was noted in the interrenal bodies of salmon killed 24 hours after exposure, but injury was not as extensive as that found in the hemopoietic tissue in the same fish (Table VIII). At exposures

TABLE VIII.
COUNTS OF CELLS IN THE INTERRENAL BODIES OF CHINOOK SALMON 24 HOURS AFTER IRRADIATION AS EYED EMBRYOS.

Dose in r	Total Number of Cells	Number of Mitotic Cells	Number of Pycnotic Cells	Percent. Mitotic Cells	Percent. Pycnotic Cells
0	747	5	0	0.67	0
250	881	3	2	0.34	0.23
500	907	3	12	0.33	1.32
1000	870	0	16	0	1.84
2500	369	0	21	0	5.70
5000	358	0	9	0	2.52
10000	340	0	26	0	7.65

of 10,000 r the percentage of cells in pycnosis averaged about 7.65 as compared with 27.21 per cent in the hemopoietic tissue. Investigations of specimens sacrificed at subsequent weekly intervals revealed that development had been arrested completely by irradiations of 2,500 r or over (Fig. 16). Some retardation of growth was noted in a few specimens irradiated with 1,000 r but no abnormalities were seen 40 days after exposure in fish given radiations of less than 1,000 r.

Spleen

In the salmon the fully developed spleen is a rounded, triangular-shaped, compact body attached to the greater curvature of the J-shaped stomach by an extension of the peritoneal investment. The spleen is probably of secondary importance to the kidney as a hemopoietic

organ in Salmonoid fishes if one can judge by the comparative sizes of the organs. Leucopoiesis and erythropoiesis occur in the spleen which in addition has the function of blood destruction, especially erythrocatheresis (Agnesotti, 1932). Hemopoietic conditions are essentially similar in kidney and spleen.

During the development of the chinook salmon from the eyed egg stage to the feeding stage the spleen not only changes its histological character but also its position relative to other organs and locality in the body cavity. At the time the chinook embryos were irradiated, the spleen was a small organ dorsal to a straight alimentary tract and definitive splenic cells were not being developed. The undifferentiated nature of the cells would indicate that the spleen did not function as a hemopoietic organ until about the time of hatching, 14 days after irradiation. As the alimentary tract develops from the more or less straight structure in the "eyed" embryo to the adult pattern in which the tract forms a complete loop at the posterior end of the stomach and duodenal region, the spleen is carried from a dorsal position in the body cavity and rotated on the greater curvature of the stomach to a ventral position in the body cavity.

The undifferentiated nature of the cells of the spleen during the "eyed" stages might indicate that the sensitivity of the organ to irradiation is different from the adult functional spleen or the kidney hemopoietic tissue. The observations below seem to bear out this assumption.

Irradiation damage to the spleen anlage 24 hours after treatment was evidenced by the presence of a few degenerate nuclei in fish irradiated with 500 r to many degenerate cells in spleen of fish given heavier doses. The presence of pycnotic, rhexic and otherwise degenerate cells accompanied a period of mitotic depression which continued for a few weeks after irradiation in the 1,000 r lot and until death in fish irradiated with 2,500 r or more. Marked histological differences from non-irradiated fish were noted in fish treated with 500 r three weeks after irradiation and occasionally seen in fish treated with 250 r during the same period. Complete recovery of the spleen seems to have taken place in four to five weeks after dosages of 250 and 500 r and in about 12 weeks after 1,000 r.

Doses of 2,500 r or greater inhibited development completely or caused some degeneration of the spleen anlage (Fig. 17). In no case,

however, was the degeneration as complete as seen in the kidney hemopoietic tissue. It would seem that the undifferentiated splenic cells were more resistant to radiation than the hemopoietic cells of the kidney.

In other respects the radiation effects on the spleen were similar to those seen in the kidney tissue. The retardation of mitotic activity, the presence of many pycnotic cells 24 hours after irradiation, with smaller numbers occurring thereafter (as long as 11 weeks in 1,000 r) and a comparative scarcity of lymphocytes in heavily irradiated spleens, followed the usual pattern as seen in the irradiation of hemopoietic tissue (Tsuzuki, 1926; Dunlap, 1942; Pohle and Bunting, 1936; Warren, 1936; etc.).

Gonads

Histological examinations indicated that no apparent injury was sustained by the gonads 24 hours after irradiation. A few degenerate nuclei were first seen in embryos nine days after exposure to 10,000 r. Some abnormal mitotic and pycnotic figures were evident in 2,500, 5,000 and 10,000 r larvae 16 to 30 days after treatment, and degenerate nuclei appeared in still greater abundance 40 to 44 days after irradiation in these groups.

Larval gonads 51 days after irradiation with 1,000 r contained fewer germ cells and fewer interstitial cells than control fish examined at this time (Table IX, Fig. 11). Fifty-eight days after treatment the gonads in fish irradiated with 250 r, 500 r and 1,000 r showed further retardation in growth or development when compared with control fish. Retardation, as measured by counting primordial germ cells, was roughly in proportion to the amounts of roentgens administered. Growth inhibition and degenerative effects continued and were accentuated towards the end of the experiment; Figures 25, 26, 27 and 28 illustrate some of the effects of irradiation 77 days after treatment with X-rays. Primordial germ cells can be recognized by large size, clear nuclei and lightly staining cytoplasm.

No attempts were made to analyze sex differences relative to radiation effects in the gonads. Some definitive sex cells seemed to be developing 93 days after irradiation (about 150 days after fertilization) in the control fish and in fish treated with 250, 500 and 1,000 r. This is later than the time of occurrence of sex differentiation in the

TABLE IX.
COUNTS* OF THE PRIMORDIAL GERM CELLS IN THE GONADS OF CHINOOK SALMON COMPARED BY DAYS AFTER EXPOSURE AND BY DOSAGE.

Days after Irradiation	Control 0 r	250 r	500 r	1000 r
9	33	42	25	27
16	46	31	64	27
23	32	35	108	39
30	14	50	46	7
37	21	47	28	42
44	32	124	79	71
51	453	83	65	53
58	2,085	287	55	25
65	1,058	683	286	47
79	6,569	380	131	67
93	7,206	247	94	69
Average	1,595.4	182.6	89.2	43.1

*Counts of over 1,000 r were arrived at by first making total counts on all five-micron sections and calculating the actual number of germ cells present using the average size of the cell nucleus (9.2 microns) and actual cell counts of the other fish as a basis. In cases where every fifth section only was used (as in the 65th, 79th and 93rd day series) actual counts were obtained by multiplying original counts by five and then correcting for size of the cell nucleus.

rainbow trout (*Salmo irideus*) which develop definitive sex cells 95 to 104 days after fertilization in water temperatures of 56° to 58° F. (Moore, 1937). The reproductive system seems more sensitive to X-rays than other organs in the chinook salmon. This is true of other animals. There are, of course, manifold considerations, and it is well to bear in mind what kind of cell changes exposure of the gonads to radiations might imply. The irradiated cell may seemingly make complete recovery and the histology may appear normal, but the fact remains that a germ cell, which, after fertilization is to produce a new individual, has been subjected to radiation, and possible submicroscopic injury may result which will be greatly magnified in the progeny.

Other Organs

Cursory histological examinations were made at weekly intervals of other organs of the chinook salmon embryo and larva, but no permanent damage could be detected in the nervous, digestive, muscular, integumentary or skeletal systems at irradiations of 1,000 r or less. The thymus gland, the pituitary gland and the auriculoventricular valve of the heart seemed to be damaged by X-rays of 1,000 r. All organs showed inhibition of development or abnormalities after irradiations of 2,500 or more.

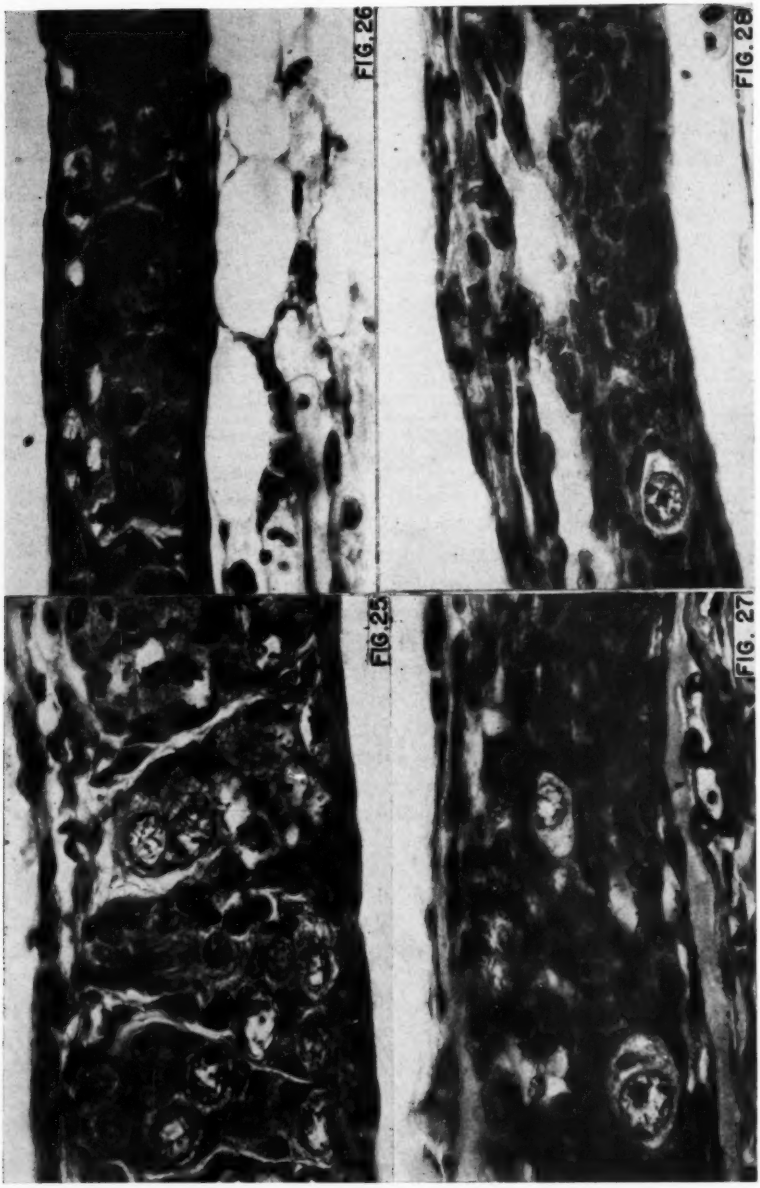


FIG. 26
FIG. 28
FIG. 25
FIG. 27

FIGURES 25, 26, 27 and 28.

Photomicrographs of five-micron sagittal sections of the gonads of chinook salmon larvae 77 days after exposures to 0 r, 250 r, 500 r and 1,000 r respectively. × 640.

Summary

An evaluation was made of the effects of X-rays on the embryos of chinook salmon, *Oncorhynchus tschawytscha* (Walbaum), using criteria such as death rate; change in growth rate, of both body weight and length; development of the eye and other organs; and edematous or other degenerative effects. Measurable effects in direct proportional relationship to the amount of roentgens administered were noted in almost all the criteria used.

"Eyed" embryos numbering 3,172 were divided into a control lot and six lots treated with 250, 500, 1,000, 2,500, 5,000 and 10,000 r at a target distance of 24 inches, using 200 kv. and 20 ma. producing 37.23 r per minute in the water in which the eggs were irradiated. Observations were made on the fish for a period of 125 days following irradiation.

Salmon given 100 per cent lethal doses of 2,500, 5,000 and 10,000 r in the eyed stages hatched and appeared normal for four weeks, then, aside from minor differences, all developed similar degenerative symptoms, and died 30 to 51 days after exposure.

Doses of 2,500 r or more arrested the development of cutaneous pigment, vascular system, fin rays and other organs. Growth in length and weight was drastically retarded. Severe vascular disturbances, lesions, loss of vitality, paralyzing effects, malformations, and edema were evident in all of these fish, being greatest just before death.

Exposure to 1,000 r retarded development of cutaneous pigment, vascular system, fins, eyes, and body length and weight. About 125 days after exposure these fish averaged 81 per cent as long and 60 per cent as heavy as non-irradiated fish. Some evidence of hemorrhage, extravasation and edema was present in a few of these young salmon. About 13 per cent of the fish died 37 to 51 days after exposure, 24 per cent 79 to 100 days after exposure and a total of 51 per cent during the 125 day period.

Doses of 500 r caused only a slight retardation of pigment development and growth in length and weight. Mortalities in these salmon and those irradiated with 250 r were slightly higher than the controls.

The syndrome resulting from irradiation was similar in young chinook salmon to that of other animals, differing mainly in speed of development of the symptoms and time of death after exposure. The latent period between exposure to minimal lethal doses and death

is longer in salmon larvae (about 40 days) than in most animals.

Histopathological studies were made on serial sections of fish killed at weekly intervals during the experiment. Studies of the kidneys, with its included hemopoietic tissue; the interrenal bodies; the spleen; the gonads and other organs of chinook salmon embryos and larvae revealed the gonads first, then the hemopoietic tissue as most sensitive to X-rays.

Counts of the primordial germ cells showed definite inhibition of development after whole body irradiations as low as 250 r with indications that a much lower dose would have some effect.

Cell counts in the hemopoietic tissue of the anterior kidney indicated temporary retardation of development at 250, 500 and 1,000 r, roughly in proportion to dose, with permanent destruction at 2,500 r. Temporary cessation of mitosis at 1,000 r and permanent cessation at higher radiations was noted, while cell destruction in proportion to the amount of dose was noted in all categories.

Counts of the glomeruli indicated slight retardation of development at 500 r and definite damage at 1,000 r. Most of the other organs examined showed some damage at 1,000 r or more.

References

1. Agnesotti, A. A. 1932. La morphologia e la genesi del sangue in avanotti di anguilla e di trota. *Arch. Zool. Ital.* **17** (3/4); 289-321.
2. Bonham, K., L. R. Donaldson, R. F. Foster, A. D. Welander & A. H. Seymour. 1948. The effect of X-ray on mortality, weight, length, and counts of erythrocytes and hematopoietic cells in fingerling chinook, *Oncorhynchus tschawytscha* (Walbaum). Growth, **12** (2); 107-121.
3. Borak, J. 1942. Radiation effects on blood vessels. I, II, III. Radiology **38** (4); 481-492; (5); 607-617; (6); 718-727.
4. Butler, E. G. 1936. The effects of radium and X-rays on embryonic development. Vol. I., Chap. XI, pp. 389-410 in Biological Effects of Radiation. Ed. by B. M. Duggar, McGraw-Hill, New York.
5. Clark, J. H. 1936. The effect of radiation on proteins. *Ibid.* Chap. VII, pp. 303-322.
6. Corbella, E. 1930. Influsso delle radiazioni Roentgen sullo svilluppo embrionale dei Teleostei (*Salmo lacustris* L., *Salmo irideus* Gibb., *Perca fluviatilis* L.). *Riv. Biol. Milano* **12**; 93-117.
7. Davison, C. & F. Ellinger. 1942. Radiation effects on nervous system and roentgen pigmentation of goldfish (*Carassius auratus*). *Proc. Soc. Exp. Biol. and Med.* **49**; (3): 491-495.
8. Dunlap, C. E. 1942. III. Effects of radiation on the blood and the hemopoietic tissues, including the spleen, the thymus and the lymph nodes. In Effects of Radiation on Normal Tissues. Ed. by S. Warren, Arch. Path. **34** (3); 562-608.
9. Ellinger, F. 1939. Note on the action of X-rays on goldfish (*Carassius auratus*). *Proc. Soc. Exp. Biol. Med.* **41** (2); 527-529.

10. ELLINGER, F. & C. DAVISON. 1942. Changes in the central nervous system of goldfish, irradiated in the depths of a water phantom. Radiology **39** (1); 92-95.

11. JORDAN, H. E. & C. C. SPEIDEL. 1924. Studies on Lymphocytes. II. The origin, function, and fate of the lymphocytes in fishes. *Jour. Morph.* **38** (4); 529-548.

12. MCCLURE, C. F. W. 1915. The development of the lymphatic system in fishes, with especial reference to its development in the trout. *Amer. Anat. Mem.* **8** (4); 1-138.

13. MARDERSTEIG, K. 1938. Über die Wirkung der Röntgenstrahlen auf die Erythropoese. II. Mitteilung. Experimentelle Untersuchungen über die Retikulozyten nach Röntgenbestrahlungen. *Strahlentherapie* **61** (1); 107-129.

14. MOORE, G. A. 1937. The germ-cells of the trout (*Salmo irideus* Gibbons). Trans. *Amer. Micr. Soc. Menasha* **56**; 105-112.

15. POHLE, E. A. & C. H. BUNTING. 1936. Histologische Untersuchungen an der Rattenmilz nach abgestuften Röntgenstrahlendosen. *Strahlentherapie* **57** (1); 121-131.

16. RUSHTON, W. 1936. Biological notes, some experiments with fry. Salmon and Trout Magazine No. 82-85; 57-67.

17. SIMPSON, G. G. & A. ROE. 1939. Quantitative Zoology, numerical concepts and methods in the study of recent and fossil animals. McGraw-Hill Book Co., New York.

18. SMITH, G. M. 1932. Eruptions of corial melanophores and general cutaneous melanosis in the goldfish (*Carassius auratus*) following exposure to X-ray. *Amer. Jour. Cancer* **16** (4); 863-870.

19. TSUZUKI, M. 1926. Experimental studies on the biological action of hard Roentgen rays. *Amer. Jour. Roentg. and Rad. Ther.* **16** (2); 134-150.

20. WARREN, S. L. 1936. The physiological effects of radiation upon organ and body systems. Vol. I., Chap. XIV, pp. 473-539. in Biological Effects of Radiation. Ed. by B. M. Duggar. McGraw-Hill. New York.

Growth, 1948, **12**, 243-254.

GROWTH IN HABROBRACON

DANIEL S. GROSCH

Department of Zoology and Entomology, N. C. State College of Agriculture and Engineering of the University of North Carolina, Raleigh, North Carolina
(Received for publication June 3, 1948)

INTRODUCTION

For over thirty years the parasitic wasp *Habrobracon juglandis* (Ashmead) has been the subject of genetic investigation by Dr. P. W. Whiting and associates (see Martin '47). In the course of these investigations many mutations have been obtained which affect the size and shape of appendages and other structures. Because of the opportunity to contrast parthenogenetically produced haploid males (the normal type) with diploid males (from planned specific matings) and females from fertilized eggs, Habrobracon promises favorable material for the study of gene dosage, expression and interaction. The logical starting point of the developmental studies (now in progress) seemed to be normal growth of the organism of which dimensional observations are presented below.

As in all holometabolous insects, increase in size, termed growth, takes place only during the larva period. The imago is constituted from the materials and the imaginal discs present when the larva pupates. For purposes of reference, larval size attained during a given time is perhaps the most useful developmental attribute in an insect like Habrobracon which has no other easily distinguishable changes during the larval period. These records as presented below furnished a reference basis in a study of dwarfism and differential mortality (Grosch '48) and are used in a histological and cytological analysis of larval growth now in manuscript.

MATERIALS AND METHODS

In order to provide offspring of known age, females were allowed to parasitize host caterpillars for one hour periods and were then transferred. In this way eggs timed to the nearest hour were pro-

cured. When the timed eggs hatched, the larvae were measured in feeding position and length and width at greatest dimension recorded. To take these measurements an ocular micrometer was used. Undisturbed larvae move very little; thus the same larva could be identified and, at desired subsequent times, measurements could be taken of it throughout larval life. The incubator used to grow all organisms was kept at 28° C.

By taking hourly measurements, parthenogenetic offspring of virgin wild type females were followed through the larval growth period. A total of 138 haploid males comprised this group of larvae. Following this haploid investigation, growth of offspring of mated females was studied by the same method. In order to be sure that the female had not run out of sperm—that is to insure a majority of fertilized eggs in the group deposited—larvae emerging from only the first twenty eggs laid by mated females were considered. Under standard laboratory conditions while the mated female has a supply of sperm in her spermatheca about two-thirds of the eggs deposited are fertilized. To make sure that the desired types of offspring were studied, the ratios of sex-types in the group were checked when measured larvae finally became adults. Experiments with offspring from mated females were of two types: (1) Ten wild type matings were planned in such a way that at least three sex-determining alleles were involved in the cross; zygotic offspring thus produced are females, (2) a cross 25c by 25 was made (7 times) in which only two sex alleles were included; therefore diploid males were produced in addition to the normal types (see Whiting '43).

Results

Data for growth of haploid larvae are given graphically in figures 1 and 2. In order to summarize results and to minimize individual variations the mean value of the group has been plotted (Richard and Kavanagh '45). The data are presented in three sets of curves which show (Fig. 1a) dimension plotted against time, (Fig. 1b) logarithm of dimension plotted against time, and (Fig. 2) increments of size gained per unit time. By expressing the information in these ways both amount of growth and rate of change of growth are represented.

The curves of increments (Fig. 2) indicate the rise and fall in

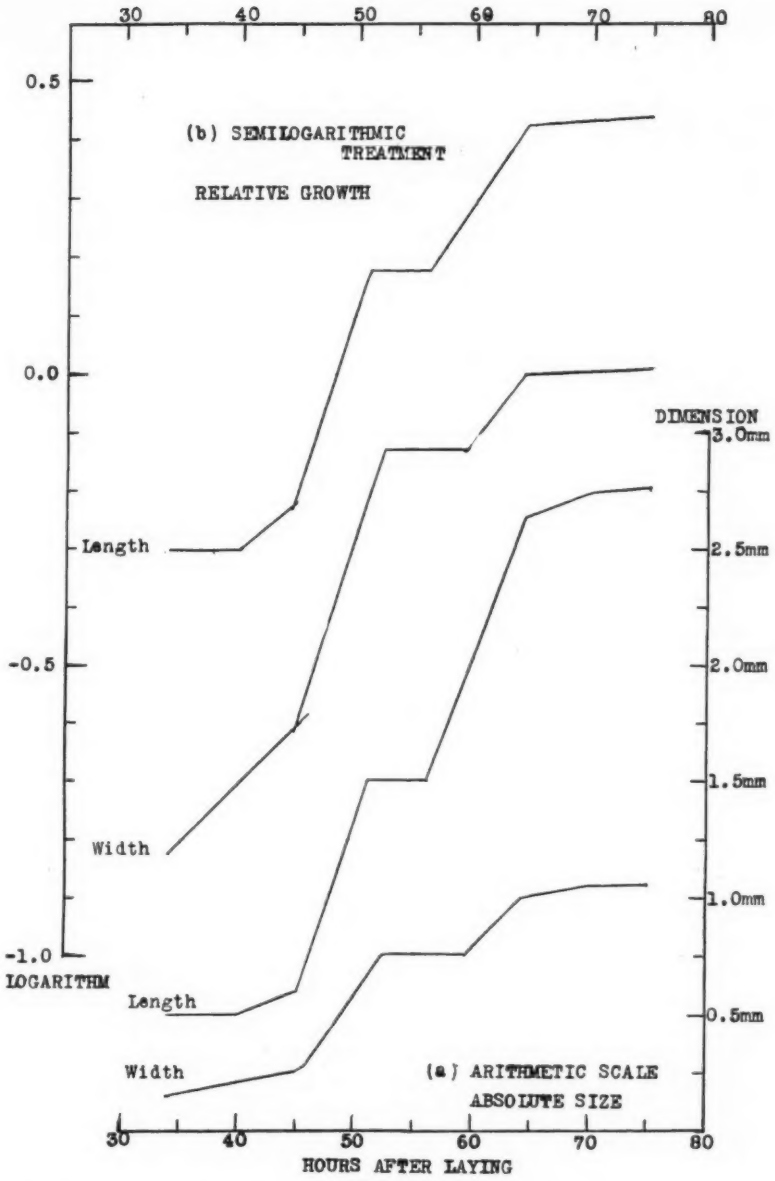


FIGURE 1

Increase in mean dimension of 138 haploid males, measuring time from the maternal deposit of eggs. (a) Dimension plotted against time. (b) Logarithm of dimension plotted aganst time.

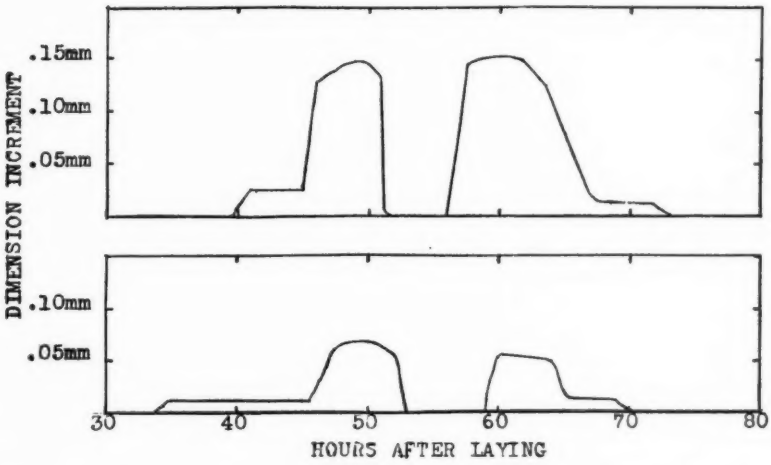


FIGURE 2

"Growth" rate curve. Increments of size gained per unit time. The upper curve represents length; the lower curve represents width.

rates of growth and the times during larva period at which these occur. The ordinary summation curves (Fig. 1a), of which the increment curves are derivatives, represent the change in absolute size of larvae during the larval period. If desired, mean size of larvae reached at a given time can be read from the pertinent point of the curves. The semilogarithmic treatment (Fig. 1b), which is preferred by many investigators who consider insect growth a problem in compound interest (Woodruff '39), indicates rate of growth by direction and slope of line and presents a basis on which to make comparison of relative growth.

Inspection of the curves of figure 1 gives the impression of four periods of growth as there are four changes in direction with three breaks in the continuity of the curves. These growth periods correspond to four stages and by careful observation moulting can be determined during each direction change in the curves. However, the shed dermis is very delicate and flakes off the larvae. In a very short time the minute flakes dry and become so inconspicuous that they cannot be distinguished from other microscopic debris such as dust and lint. The time of the second moult is interesting because of

the appreciable pause in growth. Grosch ('48) demonstrated that this is a critical developmental period.

Comparison of relative growth of length and width indicates that initially, increase in width is perceptible before increase in length, although rates of growth (as indicated by lines which are almost parellel) are essentially similar during the first and second larval periods. However, at the end of the second instar increase in width continues about an hour after length increase has ceased. During this period, the larvae in feeding position are relatively cylindrical except for the sudden taper at front and rear. The ratio of length to width at the first moult is 2.4. This ratio is maintained until just before the second moult when length increase ceases but width increase continues and the length/width ratio falls to 2.0.

In the third larval stage increase in length commences about seven hours before an increase in width is apparent. Evidently width increase does not recommence until the length/width ratio is again above 2.4. Growth in width never again exceeds growth in length and it is during the two final larval stages that a more tapered larval shape becomes pronounced, for the larval width is not maintained anteriorly. Calculated for greatest dimensions the length/width ration reaches and is maintained at 2.6.

Haploid males were chosen for initial investigations of growth in Habrobracon because from the standpoint of chromosome sets present in the organisms under consideration, a group of such offspring are more homogeneous than offspring with two or three sex types represented. Further, if development were slower or dimensions were smaller due to differences in cell size between haploid and diploid types, the lower extreme situation would be investigated first. Knowing this limit would be advantageous from the practical aspect of planning future experimental procedure. However, when data for offspring of the ten wild type matings were compared with the haploid data no outstanding differences in size or growth of larvae were apparent and curves plotted are essentially identical with those shown in figure 1 and 2. (that is without statistically significant deviation). These data are summarized to conserve space and for economy of presentation as follows:

larvae from wild type matings reached a l. of .60 mm and a w. of .25 mm at 45 hrs.
" " " " 1.50 mm " " " " .75 mm " 52 hrs.
" " " " 2.65 mm " " " " 1.00 mm " 65 hrs.
" " " " 2.75 mm " " " " 1.05 mm " 70 hrs.
at the 52 hr size there is a pause in growth of at least 5 hrs

Adults developing from these larvae totalled 116 females and 76 haploid males. Total viability was 96%; there were four inviable eggs and four inviable pupae.

Similarly critical times and sizes in offspring of two allele crosses, 25c by 25, showed no significant differences from data for haploids. Adults produced by this cross were 57 females, 32 diploid males and 16 haploid males totalling 105 animals. Twenty-eight eggs were inviable and seven individuals died between the larval and adult stages. Total viability therefore was only 75% with the greater mortality occurring with the egg.

Although growth of larvae was in close agreement, time of quitting host was variable. Some individuals moved off the host shortly after attaining full size; others remained on the host as long as five hours after attaining maximum length and width. This was observed especially in the haploids where 75 larvae quitted the host before 72 hrs (timed from deposit of the eggs), 55 quitted the host between 72 and 75 hrs and 8 quitted the host at 79 hrs. Delayed movement off host was observed for only 38 offspring of mated females with about two-thirds of these haploids. There is also variation in start of spinning after larvae have left the host. Some larvae began spinning immediately, about 20% delayed as long as 4 hours.

Discussion

Number of larval stages. As stated above, the evidence of the present report indicates four larval stages with three moults. This is consistent with the findings of Hase ('22), Schlüter ('33) and authors who have consulted this German literature. Unfortunately, the most available biological study on a braconid, Genieys '25, distinguishes only three larval stages. In view of the difficulty in identifying shed larval cuticula and the lack of easily established morphological differences to delineate stages, it is not surprising that Genieys apparently missed a moult and described one too few stages. The structures used to characterize Genieys' inclusive Stage II were the mandibles. These and other thickenings in the larval head provide characters valuable for purposes of determining the species to which a hymenopterous, parasitic larva belongs (Vance and Smith '33). However, without statistical treatment of measurements technically more exacting to obtain than dimension-of-organism-per-age, minute

structure of the braconid head does not seem reliable for identification of a specific larval stage during the middle period of growth. In any event, the number of moults should be checked by some direct method such as microscopic observation of depression slide preparations.

Unique features of size increase in parasitic hymenopterous larvae.

An S-shape has often been referred to as "the curve typical of growth" or "characteristic of simple growth phenomena". However the term growth is difficult to define and many contributing factors can be involved in this many-sided subject defined as "a lasting increase in volume during the period of progressive development". (see Heilbrunn '43). For example, in the larvae of parasitic Hymenoptera there are unique features which should be considered in any explanation of observed size increases. These features perhaps explain why summarized data show no pronounced S-shaped curves when dimension is plotted against time on an arithmetic scale.

Enlargement in larvae of parasitic Hymenoptera depends chiefly upon increase in size of the midgut. The midgut has a single opening through which the pharynx pumps materials to expand the sac-like structure. Larval tissue forms only a thin but necessarily adaptive layer around the midgut. (See figure 3). Periodic moults are needed and occur presumably when the nonliving outer cuticular layer has reached its limit of expansion. Type of material ingested seems to have no effect on expansion, for in the course of the present experiments larvae have been observed which show normal size increase while ingesting practically nothing but protozoan cysts (instead of tissue) from an infected host. When the full sized larva leaves the host and spins a cocoon, the organism is still merely a large gut surrounded by a thin layer of tissue comprised of the gut-wall, contents of the body cavity, and the body wall. In fact calculations of relative volumes indicate that in Habrobracon the volume of the midgut amounts to as much as 89% of the total larval volume. (Data of these events are included in a separate report which is a histological and cytological analysis of larval growth).

Historically, every investigator who has considered internal larval structure of parasitic wasps has described the large size of the midgut. A representative list of authors should include Hase '22, Genieys '25,

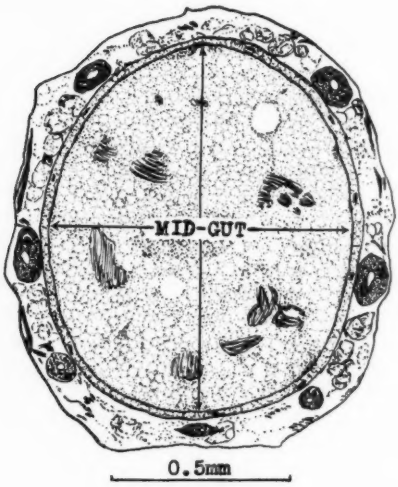


FIGURE 3

Camera lucida drawing of a typical section (prepared by the paraffin method) of a mature Habrobracon larva. Except in sections from the extreme anterior and posterior tips, the midgut occupies a very large area as shown. Other structures discernible are the ventral nerve cord, the two Malpighian tubules (smaller diameter, single nucleus, lumen often excentric), the two spinning glands (larger diameter, two nuclei, as shown, the same branch often appearing twice on a section because of the sinuous course taken), muscle fibers, and cells of the fat body.

Fink '26, Hill '26, Thompson and Parker '30, Vance '31, Cox '32, Noble '32, Vance '32, Crawford '33, Cendaña '37. These papers are typically morphological in treatment. However, there is a comprehensive paper which is not well known that has a developmental approach meriting attention. This is by Seurat '99 who emphasized the importance of the midgut as a storage pouch and argued that such an adaptation is necessary because of the limited time available to the larvae in which to attain utilizable nutrients. Observations of these investigators taken together with those of the present report, emphasize that increase in larval size depends on availability of material which can be sucked in to distend the gut. Thus a record of size increase for Habrobracon larvae is not chiefly of the multiplication of living substance. If there are limitations in ingestable material it is reflected immediately in failure to maintain normal size increase.

If larvae are forced into metamorphosis at a small size, adults which may result are dwarfed (Grosch '48).

Lack of size differences. In members of the order Hymenoptera, normal males are produced parthenogentically. These males, as exemplified in Habrobracon, have gonads which are genetically and cytologically haploid and body tissues which appear relatively haploid in comparison with equivalent tissues of zygotic individuals. When it occurs, such a difference in numbers of chromosome sets present often produces individuals which differ in size because of differences in the size of the building units, the cells (Frankhauser '45). However, in the evolution of male haploidy, special genes or gene combinations have been selected to modify the size of cells when the chromosome number is haploid (Whiting '45). In Habrobracon this enables cells of haploid males to attain the size of cells of diploid females while cells of diploid males are still larger (Grosch '45). Adjustment is such that males and females are equivalent in bodily size under favorable environmental conditions.

The present data show no differences between groups of haploid larvae and groups including both haploid and diploid larvae. If it is assumed that cell size differences in the tissue layer surrounding the constantly distending gut would be detected at the magnification used and reflected in the summarization, lack of size differences in the organisms may be interpreted as indication that the adjustment in cell size has already occurred in the embryo or in the young larva. In the case of the diploid male organism it would mean that adjustment in cell number has also occurred in view of the fact that diploid males are made up of fewer but larger cells. Early adjustments in cell size and cell number are consistant with the hypothesis that cell size differences are contributing factors in differential mortality of sex-types, since it is suspected that most diploid male offspring die before they hatch from the eggs (Bostian '35).

An interesting parallel is found in Glover's ('34) difficulty to demonstrate occurrence of two series of larvae in *Bracon* (*Microbracon*) *tachardiae,* a wasp in which he believes differences in size of male and female pupae are demonstrable. The two series of larvae overlap considerably and it is said that probably only towards the maximum and minimum of the range are larvae of one sex only. It is stated that on the average, adult males are smaller than females

although the range of size is very similar. In view of evidence from dwarfing experiments that haploid males are more viable than females when food is limited (Grosch '48) one wonders about the culture conditions in which the measured imagos were reared. If cultures were even slightly crowded, existance of size differences are explainable on an environmental basis.

Duration of last larval instar. A great deal of variation in length of last larval instar has been described in published life-cycle studies of braconids. The discharge of fecal material by the quiescent organism in the cocoon is an obvious mark of the end of the larval period and the beginning of the prepupal period. However, the time between the moult which marks the beginning of the instar and the appearance of the meconium which marks the end of the instar is not necessarily constant. Perhaps the most striking report is that of Willard ('27) with laboratory observations on *Microbracon mellitor* disclosing that time from the cessation of feeding to the start of spinning varied from 1 hour to 4 days. Therefore duration of fourth instar was anywhere from 19 to 115 hours. Willard is of the opinion that lack of suitable crevice in which to spin the cocoon is a responsible factor.

Genieys ('25) attributed variation in cocoon spinning to humidity. However Glover ('34) reaffirmed that larvae need support for cocoon construction. Glover maintained that the pith or cardboard introduced by Genieys stimulated construction of cocoons by furnishing needed mechanical support—not by drying the atmosphere.

In the present experiments delayed spinning has been observed under incubator conditions in which a maximum-minimum thermometer kept a record of temperature variations—never more than one degree—and by providing open pans of water an attempt was made to maintain humidity. Paper was always present in containers and no great movement of larvae occurred which could be interpreted as search for satisfactory spinning support. Furthermore, differences in time equivalent in duration to spinning delay were also observed in larvae quitting the host (although not necessarily in the same individual). The fact that these differences were especially noted in haploid males suggests segregation of genetic determiners for these traits.

Summary

1. Reported for Habrobracon larvae are the dimensions attained per unit time. These data are used as points of reference in developmental studies.

2. Evidence is presented for four larval stages and three moults.

3. Increase in size of larvae is related chiefly to expansion of the midgut, into which the pharynx pumps ingestible material, and not to the multiplication of living tissue.

4. Lack of size differences between groups of larvae containing haploid males, diploid males and females may indicate adjustments in cell size and cell number to have taken place as early as in the embryo.

5. There may be hereditary differences in the time at which parasitic larvae quit the host and also in the time at which larvae begin spinning cocoons.

References

1. Bostian, C. H. 1935. Biparental Males and hatchability of Eggs in *Habrobracon*. *Genetics* **20**, 280-285.
2. Cendaña, S. M. 1937. Studies on the biology of *Coccophagus* (Hym.) a genus parasitic on nondiaspidine Coccidae. *Univ. of Calif. Publications in Ent.*, **6**, 337-400.
3. Cox, J. A. 1932. *Ascogaster carpocapsae* Viereck, an important larval parasite of the codling moth and Oriental fruit moth. *N. Y. State Agr. Exp. Sta. Tech. Bull. No. 188.*
4. Crawford, A. W. 1933. *Glypta rufiscutellaris* Cresson, an ichneumonid larval parasite of the Oriental fruit moth. *N. Y. State Agr. Exp. Sta. Tech. Bull. No. 217.*
5. Fankhauser, G. 1945. Maintenance of normal structure in heteroploid salamander larvae, through compensation of changes in cell size by adjustment of cell number and cell shape. *J. Exp. Zool.*, **100**, 445-455.
6. Fink, D. E. 1926. The biology of *Macrocentrus ancylivora* Rohwer, an important parasite of the strawberry leaf roller *Ancylis comptana Froehl. J. Agr. Res.*, **32**, 1121-1134.
7. Genieys, P. 1925. *Habrobracon brevicornis Wesm. Ann. Ent. Soc. Am.*, **18**, 143-203.
8. Glover, P. M. 1934. The developmental stages of *Bracon tachardiae, Cam.* (*Hym.*) *Bull. Ent. Res.*, **25**, 521-539.
9. Grosch, D. S. 1945. The relation of cell size and organ size to mortality in Habrobracon. *Growth*, **9**, 1-17.
10. ———. 1948. Dwarfism and differential mortality in Habrobracon. *J. Exp. Zool.*, **107**, 289-314.
11. Hase, A. 1922. Biologie der Schlupfwespe *Habrobracon brevicornis* (Wesmael). *Arb. aus der Biol. Reichsanstalt. fur Land—und Forstwirtschaft*, **11**, 95-168.
12. Heilbrunn, L. V. 1943. An outline of general physiology. Second Edition Revised. W. B. Saunders Co.; Philadelphia.

13. HILL, C. C. 1926. *Platygaster hiemalis* Forbes, a parasite of the Hessian fly. *J. Agr. Res.* **32**, 261-275.

14. MARTIN, A. 1947. An introduction to the genetics of *Habrobracon juglandis* Ashmead. Hobson Book Press: New York.

15. NOBLE, N. S. 1932. Studies on *Habrocytus cerealellae* (Ashmead) a pteromalid parasite of the Angoumois grain moth *Sitrotroga cerealella* (Oliver). *Univ. of Calif. Publications in Ent.*, **5**, 311-354.

16. RICHARDS, O. W. & KAVANAGH, A. J. 1945. The analysis of growing form. Growth and Form, Essays presented to D'Arcy W. Thompson, 188-230. Oxford: Clarendon Press.

17. SEURAT, L. G. 1899. Contributions a l'etude des Hymenopteres entomophages. *Annales des Sciences Naturelles 8e, Serie*, **10**, 1-160.

18. SCHLÜTER, J. 1933. Die Entwicklung der Flügel bei der Schlupfwespe *Habrobracon juglandis Ash. Zeit. f. Morph. und Okol. der Tiere*, **27**, 488-517.

19. THOMPSON, W. R. & PARKER, H. L. 1930. The morphology and biology of *Eulimneria crassifemur*, an important parasite of the European corn borer. *J. Agr. Res.*, **40**, 321-345.

20. VANCE, A. M. 1931. *Apanteles thompsoni* Lyle, a braconid parasite of the European corn borer. *U. S. Dept. Agr. Tech. Bull. No. 233.*

21. ————. 1932. The biology and morphology of the braconid *Chelonus annulipes* Wesm., a parasite of the European corn borer, *U. S. Dept. Agr. Tech. Bull. No. 294.*

22. VANCE, A. M., & SMITH, H. D. 1933. The larval head of parasitic Hymenoptera and nomenclature of its parts. *Ann. Ent. Soc. Am.* **26**, 86-94.

23. WHITING, P. W. 1943. Multiple alleles in complementary sex determination of Habrobracon. *Genetics*, **28**, 365-382.

24. ————. 1945. The evolution of male haploidy. *Quart. Rev. Biol.*, **20**, 231-260.

25. WILLARD, H. F. 1927. Parasites of the pink bollworm in Hawaii, *U. S. Dept. Agr. Tech. Bull. No. 19.*

26. WOODRUFF, L. C. 1939. An analysis of insect growth curves. *N. Y. Ent. Soc. J.*, **47**, 47-55.